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ORIGINAL ARTICLE

Dieckol Attenuates Cell Proliferation in Molt-4 Leukemia Cells via Modulation of JAK/STAT3 Signaling Pathway

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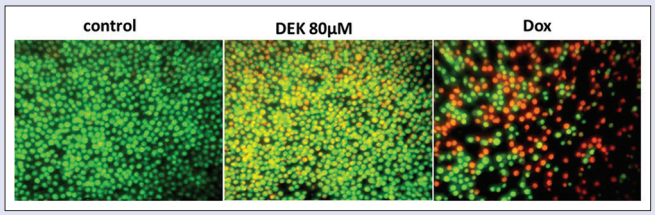
ABSTRACT

Background: Leukemia is a cancer of the hematopoietic stem cells, which leads to an uncontrolled proliferation of leukocytes in blood. It is responsible for one of the most important cancer-associated deaths across the globe. **Materials and Methods:** In this study, we analyzed whether dieckol (DEK), a polyphenolic compound obtained from brown algae, can suppress cell proliferation via regulation of JAK/STAT3 signaling pathway in leukemia cell lines (Molt-4). **Results:** According to our results, DEK induced cytotoxicity, altered the cell morphology, caused nuclear damage, enhanced the formation of reactive oxygen species, decreased the production of mitochondrial membrane potential, reduced the levels of antioxidants (reduced glutathione, catalase, and superoxide dismutase), and augmented the level of thiobarbituric acid reactive substances in Molt-4 cell lines. Furthermore, STAT3 has been recognized as an important transcriptional mediator that controls cell proliferation. Thus, suppression of STAT3 transcription is a novel approach for the suppression of Molt-4 cell proliferation. In this study, DEK inhibited STAT3 translocation, thereby suppressing the increased expression of cyclin E1, PCNA, cyclin D1, and JAK1 in Molt-4 cell lines. **Conclusion:** In summary, DEK suppressed the cell proliferation of Molt-4 cells via inhibition of JAK/STAT3 signaling pathway.

Key words: Cell proliferation, dieckol, JAK/STAT3 signaling, leukemia, Molt-4 cells

SUMMARY

- Oncogenic transcriptional mediator of STAT3 is the major target for the management of leukemia
- DEK suppresses leukemia cell growth and proliferation via suppression of JAK/STAT3 signaling pathway in Molt-4 cells.



Abbreviations used: DEK: Dieckol; DOX: Doxorubicin; SOD: Superoxide dismutase; MMP: Mitochondrial membrane potential; ROS: Reactive oxygen species.

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INTRODUCTION

Leukemia is a cancer of the hematopoietic stem cells that leads to uncontrolled proliferation of leukocytes in blood. It is one of the most important cancer-associated deaths across the globe.[1,2] The abnormal accumulation of immature T-lymphoblast leads to cancer formation and uncontrolled proliferation of the myeloid cells in the bone marrow.[3] Leukemia accounts for nearly 5% of all the cancers and ranks sixth among the different human malignancies. It is a common childhood neoplasm resulting in the death of around 30% of the children and adolescents under age of 14 years.[4]

Conventional therapies such as chemotherapy, surgery, radiation, and immune therapy have limited success in curing leukemia; therefore, there is an urgent need for alternative approaches in cancer treatment. Therefore, efforts are being made to obtain natural anticarcinogens that could slow, reverse, or even prevent cancer development. Plants play a significant role in the treatment of carcinogenesis.[5] Some of the plant-derived drugs comprise more than 50% of all the anticancer drugs in the market. Many investigational studies have tested conventional plants in an attempt to obtain new curative drugs that have less toxic side effects.[6] One such compound is dieckol (DEK), a polyphenolic agent obtained from brown algae *Ecklonia cava*.[7] It shows anti-inflammatory, antihyperlipidemic, anti-aging, antineurodegenerative, antitumor, antiallergic, and antidiabetic activity.[8,9] Furthermore, it suppresses human hepatic, breast, and ovarian carcinoma cell proliferation.[10-12]

Oncogenic transcriptional mediator of STAT3 has been frequently targeted for the management of leukemia. JAK biomarkers are frequently found to be mutated in proliferative neoplasms that are responsible for the constitutive stimulation of JAK/STAT3 signaling

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pathway.[13] This, in turn, leads to uncontrolled cell proliferation by increasing the levels of key transcriptional proteins containing cyclins D1 and E1 and PCNA, which are involved in the regulation of cell cycle.[14] Thus, inhibition of JAK/STAT3 signaling pathway has been regarded as the central target in cancer therapy.[15] So far, no studies have reported on the suppression of JAK/STAT3 signaling pathway by DEK in leukemia. Therefore, in this study, we aimed to examine the role of DEK in the suppression JAK/STAT3 signaling on leukemia cell lines (Molt-4).

MATERIALS AND METHODS

Reagents and cell culture

DEK, doxorubicin (DOX), and 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide (MTT) were procured from Sigma-Aldrich Co., Ltd. Antibodies for cyclins D1 and E1, PCNA, JAK1, and STAT3 were obtained from Cell Signaling Technology. Human leukemia cell line (Molt-4) was cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum and antibiotics (streptomycin, penicillin, and ampicillin 100 units/mL each) in a humidified atmosphere with 5% CO_2 at 37°C.

Cytotoxic assay and morphological study

The cytotoxic effects of DEK on Molt-4 cells were analyzed via MTT analysis. Briefly, Molt-4 cells were cultured in 24-well plates (1×10^4 cells/well) at 37°C for 24 h. After 24 h, different concentrations of DEK (5, 10, 20, 40, 80, and 160 μM) were added to the culture medium and incubated for 24 h. Then, the medium was removed and 10 μL of 0.5 mg/mL MTT solution was added to all the wells and incubated for the next 4 h. The formazan crystals formed were dissolved by the addition of 100 μL of dimethyl sulfoxide and finally the absorbance was measured at 570 nm using an ELISA plate reader, USA. In addition, the morphology of Molt-4 cells was observed by the phase contrast microscope, USA.[16]

Analysis of acridine orange/ethidium bromide staining

Molt-4 cells were seeded in 6-well plates (0.6×10^6 cells/well) and were incubated at 37°C for 24 h. Then, DEK (40 and 80 μM) and DOX were added and the cells were again incubated for 24 h. After incubation, 10 μL of cell culture medium was loaded on the glass slide and the cells were stained using acridine orange/ethidium bromide (AO/EB). The cells were covered with a cover slip and observed under the fluorescent microscope (NIKON Eclipse 80i, Japan).[17]

Estimation of reactive oxygen species and measurement of mitochondrial membrane potential

Molt-4 cells were incubated at 37°C for 24 h. After this, various concentrations of DEK (40 and 80 μM) and DOX were added and again incubated for 24 h. After that, the media was decanted and 5 μM of 2,7-dichlorofluorescein diacetate (DCFH-DA) dye was added to estimate the amount of reactive oxygen species (ROS) formed. For the measurement of mitochondrial membrane potential (MMP), Rh-123 was added and the changes were investigated under the laser scanning confocal microscope (NIKON Eclipse 80i, Japan).

Assays of lipid peroxidation and antioxidants

Molt-4 cells were added at different concentrations (40 and 80 μM) of DEK and DOX and incubated at 37°C for 24 h. Then, the cells were washed, lysed, and centrifuged at 12000 rpm (15 min) at 4°C. The supernatant was used to estimate the parameters responsible for oxidative stress. The level of superoxide dismutase (SOD) was investigated by the method described by Kakkar *et al.*[18] The concentration of glutathione (GSH) was estimated by the method of Ellman (1959).[19] The thiobarbituric acid reactive substances (TBARS) were measured by the method described by Ohkawa *et al.*[20] Finally, the catalase (CAT) levels were determined by the method described by Sinha.[21]

Western blot analysis

Cells were grown for 24 h and DEK (40 and 80 μM) and DOX were added to the wells and incubated at 37°C for 24 h. Then, the cells were lysed with RIPA buffer as explained previously.[22] Proteins (40 μg) were separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis technique and the separated proteins were transferred onto polyvinylidene fluoride membranes. Then, the proteins were blocked with skimmed milk (5%) in TBST and incubated with particular primary antibodies (cyclins D1 and E1, PCNA, JAK1, and STAT3) for overnight at 4°C. Afterward, the membranes were incubated with secondary antibodies for 2 h at room temperature (RT). Protein bands were observed using enhanced chemiluminescence detection kit (Bio-Rad, USA).

Statistical analysis

The data were analyzed as mean ± standard deviation. Statistical analysis was conducted using the GraphPad Prism 8 software (San Diego, CA, USA). We performed analysis of variance and Tukey's test as a *post hoc* analysis. The differences in mean were found to be significant if $P < 0.05$.

RESULTS

Effect of dieckol on the inhibition of Molt-4 cell proliferation and morphological changes

Cell viability of Molt-4 cells was assessed after treating the cells with DEK at different concentrations (5, 10, 20, 40, 80, and 160 μM) via MTT assay [Figure 1]. According to the results, DEK caused a dose-dependent decrease in the cell number (IC_{50} = 80 μM); therefore, for further experiments, we selected a concentration of 40 and 80 μM. In addition, we examined the morphological changes using the phase contrast microscope. In contrast to the control cells, DEK (40 and 80 μM) and DOX-treated cells exhibited marked changes

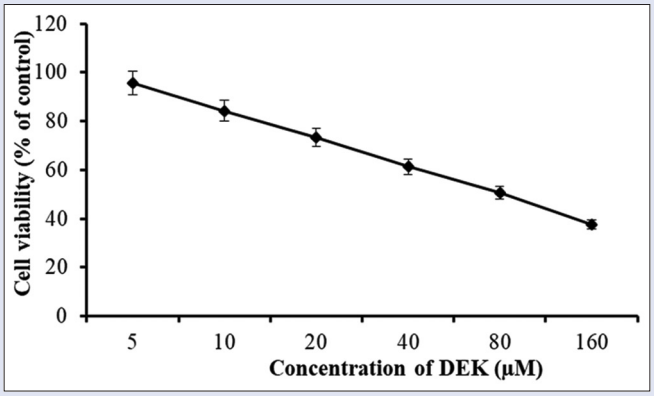

Figure 1: The cytotoxic effect of dieckol on Molt-4 cells was measured by 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide assay. Figure 1 proves the significant cytotoxicity of dieckol against the Molt-4 cells. Among the different concentrations (5–160 μM), the 80 μM of dieckol was inhibited 50% of cell growth (IC_{50})

in the cellular morphology, such as reduced cell density and condensed cell shape [Figure 2].

Effect of dieckol on the morphology of apoptotic cells by acridine orange/ethidium bromide staining

We detected the fluorescence of the apoptotic cells by performing AO/EB staining [Figure 3]. According to the results, untreated control cells did not display any red/yellow fluorescence, which shows that there was no apoptosis and nuclear damage. However, cells that were treated with 40 and 80 μM of DEK and DOX revealed red/yellow fluorescence, which points toward the initiation of the apoptotic pathway.

Determination of reactive oxygen species in Molt-4 cells

The effect of DEK (40 and 80 μM) on the production of ROS in Molt-4 cells was analyzed via fluorescence microscopy [Figure 4]. According to our results, DEK significantly augmented the levels of ROS in Molt-4 cells. Control cells revealed low levels of ROS in Molt-4 cells. DOX also enhanced the production of ROS compared to the control cells.

Effect of dieckol on mitochondrial membrane potential levels in Molt-4 cells

The effect of DEK (40 and 80 μM) on the Molt-4 cells was analyzed by fluorescence microscopy [Figure 5]. According to the results, DEK caused a substantial reduction in the MMP levels of Molt-4 cells. Control cells revealed enhanced levels of MMP in Molt-4 cells. DOX treatment also decreased the levels of MMP in comparison to the control cells.

Effect of dieckol on lipid peroxidation and antioxidant levels

The effect of DEK on the levels of antioxidants such as SOD, GSH, and CAT and lipid peroxidation indicators such as TBARS was estimated [Figure 6]. TBARS levels were found to be decreased and SOD, GSH, and CAT levels were increased in Molt-4 cells. In contrast, compared to the control cells, there was a high level of TBARS and low levels of SOD, GSH, and CAT in DEK- (40 and 80 μM) and DOX-treated Molt-4 cells.

Effect of dieckol on protein expression

In order to recognize the molecular mechanisms responsible for the reduction in cell proliferation, we measured the proteins that are essential for these biological functions [Figures 7 and 8]. We found that the expression of STAT3, JAK1, PCNA, and cyclins D1 and E1 was downregulated after incubating the Molt-4 cells with DEK (40 and 80 μM) and DOX. However, their expression was found to be upregulated in control cells.

DISCUSSION

Nowadays, researchers are interested in nutritional and remedial phytochemical derivatives from natural sources as a healthy alternative for the detection of novel anticancer drugs.[23] DEK is an anticancer drug, which inhibits cell proliferation of various types of cancer cells. Cell viability of Hep3B cells was found to be downregulated after DEK treatment, with an IC_{50} value ranging between 80 and 100 μM.[24] Another study has confirmed that DEK reduces cell viability in SKVO3 cells with an IC_{50} range of 80–120 μM.[12] Herein, we confirmed that DEK inhibited the proliferation of Molt-4 cells in a dose-dependent manner. We also confirmed morphological analysis and nuclear damage by dual staining. The phase contrast micrographic image demonstrates that DEK can reduce cell density and alters the cellular shape. The morphological changes are mainly induced by the excessive production of intracellular ROS. This result coincides with a previous research.[24] Another study reported that DEK suppressed cell proliferation and nuclear damage in Hep3B cells.[25]

The mitochondrion is one of the central imperative organelles in controlling cell death in addition to other indicator in apoptosis.[26] ROS are molecules with unpaired valence shell electrons and are therefore called as free radicals; they are extremely active and cause a significant amount of oxidative damage to the cell.[27] Increased levels of ROS are considered to be responsible for causing cell death. According to our results, DEK increased the levels of ROS in Molt-4 cells. Loss of MMP inhibits cell proliferation and causes apoptotic cell death.[28] Modification of MMP in DEK-treated Molt-4 cells might be due to the failure in the production of ATP, which leads to either necrosis or apoptosis.[29] In this study, depleted levels of MMP and elevated levels of ROS show that DEK stimulated apoptosis in Molt-4 cells. These results are in accordance with previous reports that DEK inhibited cell proliferation by increasing the ROS formation and decreasing the levels of MMP in SKOV3 cell lines.[12] Oxidative stress is explained as an imbalance between the formation of ROS and reduced antioxidant defense systems.[30] This imbalance can lead to mutagenesis or even lead to cell death and increased levels of cell proliferation regarding of its quantities were evaluated to antioxidants such as SOD, CAT, and GSH in the cells. It has been reported that an imbalance in the levels of intracellular ROS can provoke cell cycle arrest and suppression of cell proliferation.[31] In this study, increased level of TBARS and reduced antioxidant levels were recorded after incubating the cells with DEK. Similar results have been reported by Sivagami *et al.*[32] after treating with HT-29 cells with hesperetin.

Normally, the overexpression of STAT3 leads to the activation of key transcription proteins responsible for proliferation. The activated STAT3 has been implicated in the cell survival and proliferation in leukemia.[33-35] In this study, STAT3 and JAK1 protein expression was notably downregulated at increased levels of DEK. These genes might be the primary target to stimulate apoptosis in leukemic cells.[36] In a

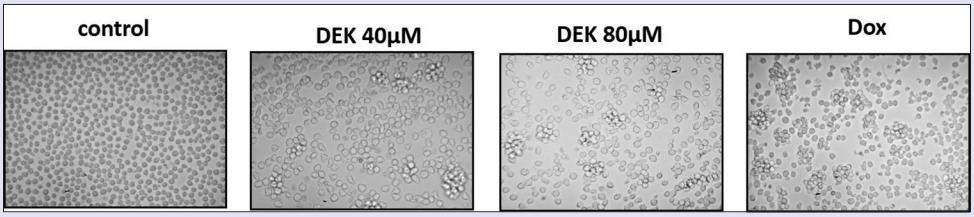

Figure 2: Dieckol suppresses the proliferation of Molt-4 cells. Cellular morphology was viewed under a phase contrast microscope. The treatment with the 80 μM of dieckol was significantly inhibited the cell viability of Molt-4 cells, as seen in the doxorubicin treatment

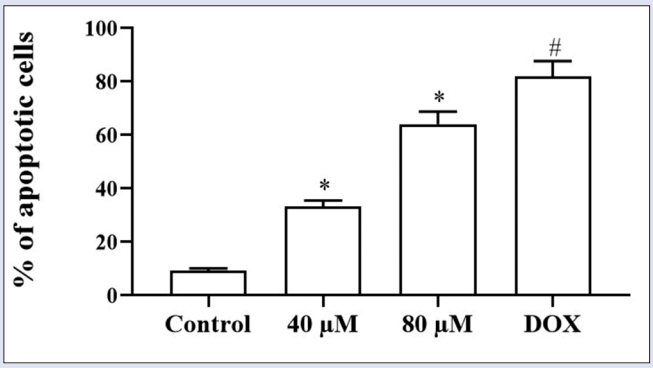

Figure 3: Dieckol on apoptotic morphological changes was analyzed by dual staining (acridine orange/ethidium bromide). (a) Microscopy images of control cells were showed green fluorescence, whereas dieckol- and doxorubicin-treated cells showed red/yellow fluorescence. (b) Bar diagram showed that the percentage of apoptotic cells was calculated. The data represent mean ± standard deviation of triplicate, $^{*}P < 0.05$ and $^{\#}P < 0.01$ as compared with the control group

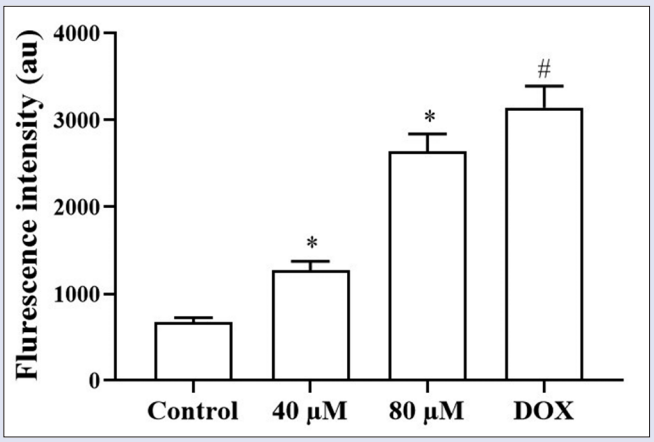

Figure 4: The effect of dieckol on intracellular ROS generation was determined in Molt-4 cells using 2,7-dichlorofluorescein diacetate staining. (a) Fluorescence microscopic image for dieckol on intracellular reactive oxygen species generation. (b) Percentage of reactive oxygen species generation was detected by spectrofluorometer. The data represent mean ± standard deviation of triplicate, $^{*}P < 0.05$ and $^{\#}P < 0.01$ as compared with the control group

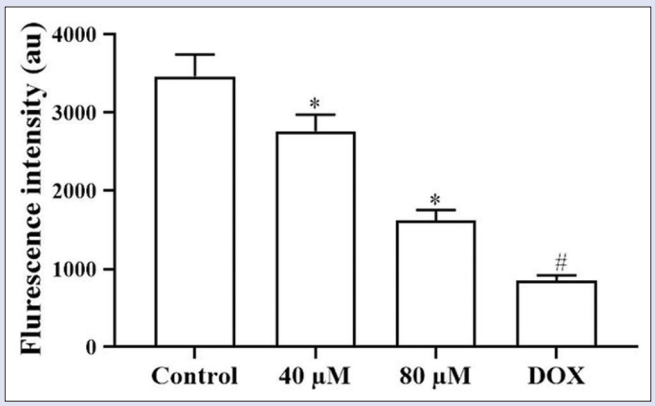

Figure 5: The effect of dieckol on mitochondrial membrane potential status was determined in Molt-4 cells using Rh-123 staining. (a) Fluorescence microscopic image for dieckol on the status of mitochondrial membrane potential. (b) Percentage of mitochondrial membrane potential was detected by spectrofluorometer. The data represent mean ± standard deviation of triplicate, $^{*}P < 0.05$ and $^{\#}P < 0.01$ as compared with the control group

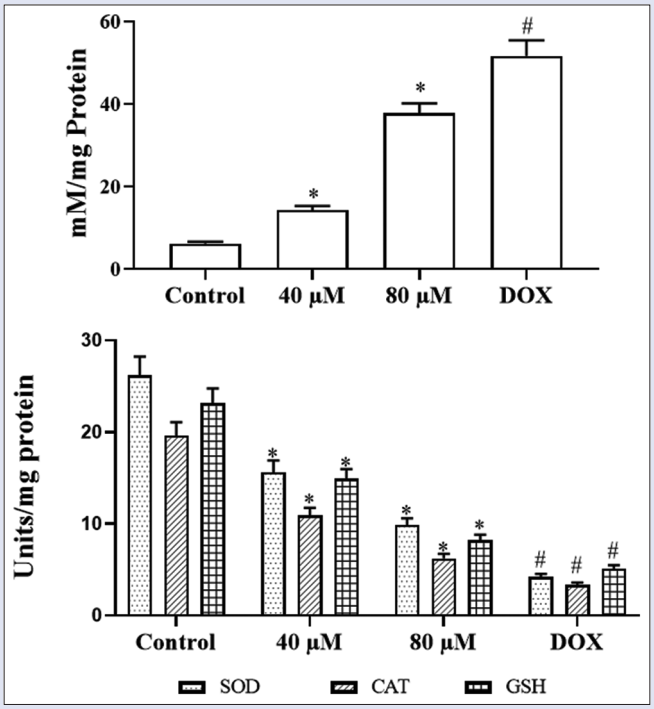

Figure 6: Dieckol enhanced lipid peroxidation and modulate cellular antioxidant status in Molt-4 cells. The data represent mean ± standard deviation of triplicate, $^{*}P < 0.05$ and $^{\#}P < 0.01$ as compared with the control group. Catalase-mM of hydrogen peroxide consumed per minute. Superoxide dismutase-enzyme concentration required for 50% inhibition of nitroblue tetrazolium reduction in 1 min. Glutathione-mg/dl

previous study, DEK inhibited the activation and nuclear translocation of STAT1 in HaCaT cells, which supports our data.[37] Uncontrolled cell proliferation can be stimulated by altered expressions of protein associated with the cell cycle. So far, several anticancer drugs have demonstrated to induce apoptosis in cancer cells via blocking of cell proliferation and cell cycle arrest.[38] Cyclin D1 is one of important controllers of cell cycle development. Cyclin E1 and PCNA are key markers of proliferation including in leukemia. These markers have been reported to be overexpressed in various cancer cell lines.[39-42] In this study, we found that DEK downregulated the expression of cyclins D1 and E1 and PCNA in Molt-4 cells. Previous studies also reported that DEK downregulated the expression of these mediators in 3T3-L1 preadipocytes[43] and A549 cells.[44] These data explain the anticancer potential of DEK against leukemia; however, additional research is still needed to find the exact therapeutic potential of DEK.

CONCLUSION

In this study, we observed that DEK induced cytotoxicity, altered cell morphology, damaged nuclear integrity, enhanced ROS production,

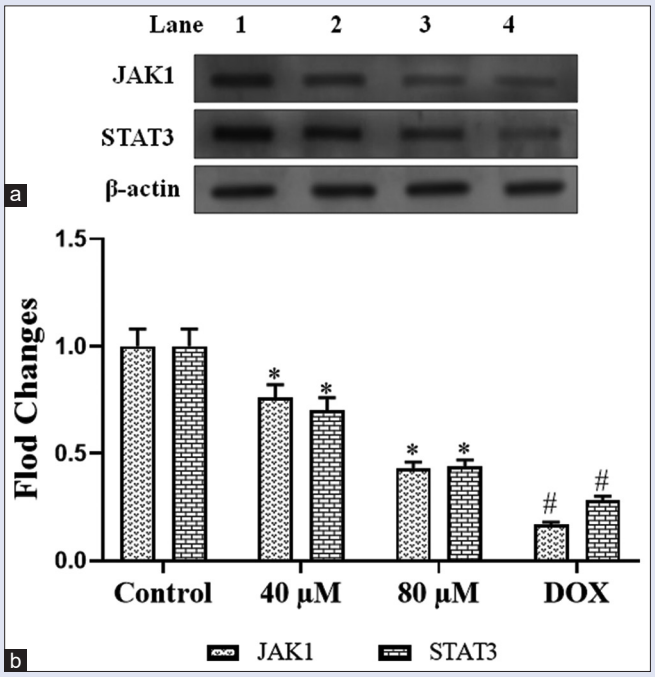

Figure 7: Dieckol inhibits JAK/STAT3 translocation in Molt-4 cell lines. (a) Western blots images for JAK1 and STAT3 in Molt-4 cell lines. Lane 1: Control; Lane 2: dieckol (40 μM); Lane 3: dieckol (80 μM); Lane 4: doxorubicin. (b) The densitometry data were analyzed interest of proteins normalized to respective β-actin loading control. The data represent mean ± standard deviation of triplicate, $^{*}P < 0.05$ and $^{\#}P < 0.01$ as compared with the control group

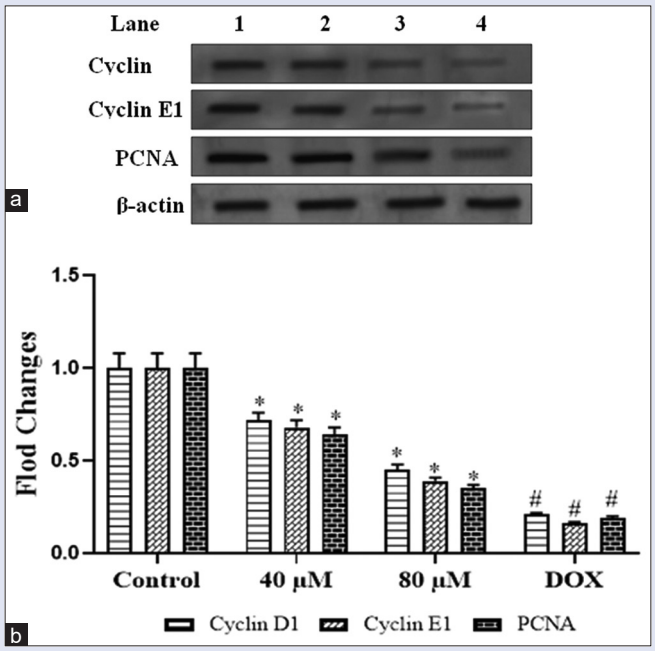

Figure 8: Dieckol inhibits cell proliferation in Molt-4 cell lines. (a) Western blot images for cyclin D1, cyclin E1, and PCNA cell proliferative markers in Molt-4 cell lines. Lane 1: control; Lane 2: dieckol (40 μM); Lane 3: dieckol (80 μM); Lane 4: doxorubicin. (b) The densitometry data were analyzed interest of proteins normalized to respective β-actin loading control. The data represent mean ± standard deviation of triplicate, $^{*}P < 0.05$ and $^{\#}P < 0.01$ as compared with the control group

decreased MMP, reduced antioxidants (GSH, CAT, and SOD), and augmented TBARS in Molt-4 cell lines. Furthermore, DEK treatment inhibited STAT3 translocation, thereby suppressing the overexpression of cyclins D1 and E1, PCNA, and JAK1 in Molt-4 cells. This result shows that DEK suppresses leukemia cell proliferation via suppression of JAK/STAT3 signaling pathway in Molt-4 cells.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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