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ORIGINAL ARTICLE

Davanone terpenoid inhibits cisplatin-resistant acute myeloid leukemia cancer cell growth by inducing caspase-dependent apoptosis, loss of mitochondrial membrane potential, inhibition of cell migration and invasion and targeting PI3K/AKT/MAPK signalling pathway

Yi Xiao, Taoran Deng, Di Wang

Department of Hematology, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430030, China

Summary

Purpose: *Acute myeloid leukemia (AML) is the most frequent leukemia identified in 25% of adults and in 15-20% of children. In the current study, the cytotoxicity and apoptosis-inducing properties of davanone - a terpenoid, were examined on human AML cell line NCI-H526 and normal AML-193 cell line.*

Methods: *The cytotoxic effects were examined through MTT assay and apoptosis was studied through DAPI and Annexin V/PI staining. Further, the effect on MMP and ROS levels were investigated through flow cytometry. Cell migration and invasion were determined by wound healing and cell invasion assays respectively. Western blotting analysis was performed to study the expressions of apoptosis and PI3K/AKT/MAPK signalling pathway associated proteins.*

Results: *The results revealed that Davanone induced cytotoxicity in NCI-H526 cells in a dose-dependent manner without causing too much toxicity to the normal AML-193 cells. Further investigations were done in order to validate whether the cytotoxicity was apoptosis-mediated, and the results revealed that cytotoxicity of the test molecule was apoptosis-dependent. On further investigations through western blotting analysis, cytotoxicity was shown to be due to caspase-dependent apoptosis with increased expressions of caspase-3 and Bax and decreased expressions of Bcl-2. Next, it was seen that Davanone treatment led to decrease in mitochondrial membrane potential (MMP) and an increase in reactive oxygen species formation (ROS). The tested molecule also significantly suppressed cell invasion and migration of leukemia cells. Finally, the effect on PI3K/AKT/MAPK signalling pathway was examined and the expression of related proteins was altered significantly.*

Conclusions: *The present study outcomes propose that Davanone terpenoid could be considered as a promising anticancer agent for AML.*

Key words: *davanone, acute myeloid leukemia, flow cytometry, apoptosis, cell migration*

Introduction

Acute myeloid leukemia (AML) - a heterogeneous hematopoietic malignancy-arises from deregulation of cell differentiation, cell proliferation and cell death caused by epigenetic and genetic alterations in hematopoietic progenitor or stem cells [1,2]. AML is the most frequent type of acute leukemia prevailing in adults and near about 80% of acute leukemia patients suffer from AML. The incidence of AML per 10 million patients suffering from this disease is 1.3% and 12.2% for the age

Corresponding author: Department of Hematology, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430030, China.
Tel & Fax: +86 027 63639830, Email: yixiao@tjh.tjmu.edu.cn
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groups up to 65 and above 65 years, respectively [3]. According to American Cancer Society, in United States alone there were around 60,000 new cases and 25,000 deaths expected of leukemia in 2016, including 19,950 AML cases with 10,400 deaths [4]. Even though chemotherapy results in complete remission of this malignancy in many cases, its lethality gets enhanced due to high relapsing rate [5]. Reoccurrence causes declination in 4-year overall survival rate, which is 40% after diagnosis [6]. Accruing evidence specifies that partial abolition of abnormal drug-refractory and self-renewal leukemia stem cells in bone marrow niche sites is accountable for AML relapsing [7]. Moreover most of the patients develop resistance to chemotherapy hence limiting the refractory chemotherapeutic options [8,9]. Thus to completely curb AML new novel approaches and therapeutic agents are the need of the hour. For the past 50 years natural products have remained the backbone of cancer chemotherapy. At present, three quarters of available drugs are natural products or linked to them [10]. In 2000, out of all anticancer drugs which were in clinical trials, 57% were natural products or derived from them [11]. Terpenoids are renowned as a vast group of natural compounds having a comprehensive range of pharmacological as well as biological activities [14,15]. Terpenoids show antimicrobial, antibacterial, antiseptic, analgesic, anti-inflammatory, carminative, anti-spasmodic and anticancer properties [16-18]. Davanone is a terpenoid isolated from Davana oil of Artemisia pallens, Wall, a flowering herb belonging to the family Compositae. Herein, we evaluated the Davanone molecule for its inhibitory effects on cisplatin-resistant AML cell growth by inducing caspase-dependent apoptosis, loss of mitochondrial membrane potential, inhibition of cell migration and invasion and targeting PI3K/AKT/MAPK signalling pathway

Methods

MTT cell proliferation assay

Davanone molecule action on AML cells was estimated through MTT ((3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide tetrazolium) reduction assay. Briefly, AML cell line NCI-H526 as well as normal AML-193 cell line were trypsinized and graded at a density of 5×10^3 cells/well onto 96-well plates (Corning, USA). Both the cell types were cultured overnight and then the used RMPI-1640 medium was replaced with fresh one containing different doses of the test molecule (control, 0.78, 1.56, 3.12, 6.2, 12.5, 25, 50, 100 and 200 μM) for 24 h. Then, 20 μl of MTT (Sigma-Aldrich, USA) at a concentration of 5 mg/ml dissolved in phosphate buffered saline (PBS) was added to each well plate. Viable cells bear NAD(P)H-dependent oxidoreductase enzymes which reduce the MTT to formazan crystals. These crystals were seen after incubating the well plates for 4h at 37°C after addition of MTT solution. After removal of the precipitate, crystals were liquefied in 100 ml DMSO. The optical density (OD) was assessed at 490 nm through a microplate reader (Bio-Tek, USA).

Apoptosis investigation through DAPI staining and Annexin V/PI assay

Using 96-well plates, AML cells were harvested at a density of 5×10^2 cells/well. These cells were then exposed to different doses of Davanone molecule (control, 5, 10 and 20 μM), and incubation was performed for 24h at 37°C. Next to incubation these cells were stained with 4',6-diamidino-2-phenylindole (DAPI) which was followed by further incubation for 15 min at 37°C. Next, washing and fixation of target cells was accomplished with PBS and 10% formaldehyde respectively. The examination of DAPI-stained blue colour cells was done under fluorescence microscope. Analogous method was performed for annexin V/PI assay except using annexin V/PI for staining and observations were made via flow cytometry.

Measurement of the mitochondrial membrane potential (MMP) and intracellular ROS generation

Flow cytometer (Beckman-Coulter Co., USA) and fluorescent dye Rh123 (cell permeable cationic dye) were used to detect target molecules effect on MMP of AML cell line NCI-H526. Rh123 (100 μl) was added to NCI-H526 cell culture for 50 min at 37°C. Assembling of the cells was performed by pipetting followed by washing twice with PBS. Cells were investigated at a density of 10^4 per sample by flow cytometry.

For ROS detection, cells were collected by pipetting and subjected to washing with PBS. Washing was followed by addition of 1 μM dihydrorhodamine 123 (DHR) for 60 min at 37°C. These cells were then double-washed with PBS. NCI-H526 cell cultures at a density of 10^4 cells per sample were investigated for ROS detection by means of flow cytometry.

Wound healing assay

NCI-H526 cells were exposed to varying doses of Davanone (control, 5, 10 and 20 μM). After exposure, the RMPI-1640 medium was decanted and cells were washed with PBS. A wound was scratched in each well using a sterile pipette tip and once more cells were washed. Before culturing of cells for 24h a photograph was captured and the same was repeated after culturing, with an inverted microscope with 100× magnification (Leica, Germany).

Cell invasion assay

Cell invasion suppression ability of Davanone on NCI-H526 cells was estimated by transwell chambers assay with Matrigel. After treatment with varying dose of target molecule (control, 5, 10 and 20 μM), the bottom chambers were filled only with medium and the upper chambers were filled with around 200 ml of cell culture. Afterwards, chambers were incubated for 24h which was followed by fixation with methyl alcohol, and later crys-

tal violet was used for staining. Finally, invaded cells were calculated at 200× magnification with inverted microscope (Leica, Germany).

Western blotting analysis for determination of protein expressions

RIPA lysis buffer (ThermoFisher Scientific) containing the protease inhibitor was utilized for lysing the treated NCI-H526 cells. From each sample about 35 µg of proteins were separated, followed by transference to polyvinylidene difluoride (PVDF) membrane. Thereafter, fat-free milk was used for blocking these membranes at room temperature for 60 min. Next, NCI-H526 cells were treated with primary antibodies overnight at 4°C. Accordingly, secondary antibody treatment was performed which was followed by 1-h incubation. Finally, Actin was used for normalisation and for signal detection Odyssey Infrared Imaging System was used.

Statistics

All the data is presented as mean ± SD. The significance levels for comparison of differences were assessed by one-way ANOVA, followed by Dunnet *post hoc* test for multiple comparisons (Graph-Pad Software, USA). $P<0.05$ was considered statistically significant.

Results

Determination of cytotoxicity of Davanone on normal and AML cells

MTT assay was performed in order to determine the effect of Davanone (Figure 1A) on cell viability of both acute myeloid leukemia cancer cell line NCI-H526 as well as normal AML-193 cell line. The results depicted that the test molecule inhibited the cell viability of leukemia cells significantly without considerably altering the viability of normal cell line in a concentration-dependent manner. The viability of leukemia cells reduced from 100% to almost 0% after treatment with varying doses of the test molecule (control, 0.78, 1.56, 3.12, 6.2, 12.5, 25, 50, 100 and 200 mmol/L) (Figure 1B). The IC_{50} value of Davanone against leukemia cells was much less as compared to normal AML-193 cells, indicating its selective cytotoxicity in leukemia cells.

Davanone induces apoptosis in AML cell line NCI-H526

Estimation of apoptosis induction by the test molecule was performed with DAPI and annexin V/PI assay through fluorescence microscopy and flow cytometry respectively photographs were captured at different doses (control, 5, 10 and 20 µM). DAPI investigation signified that exposure of target cells to test molecule exclusively increased the percentage of apoptotic cells on increasing the drug dose. Davanone-treated cells exhibited disrupted, fragmented, reduced and contracted nuclei, high fluorescence as well as randomly distributed chromatin in comparison to negative control cells which showed smooth, regular nucleus with normally dispersed chromatin and decreased fluorescence (Figure 2). Annexin V/PI staining quantified the number of apoptotic cells and showed the tremen-

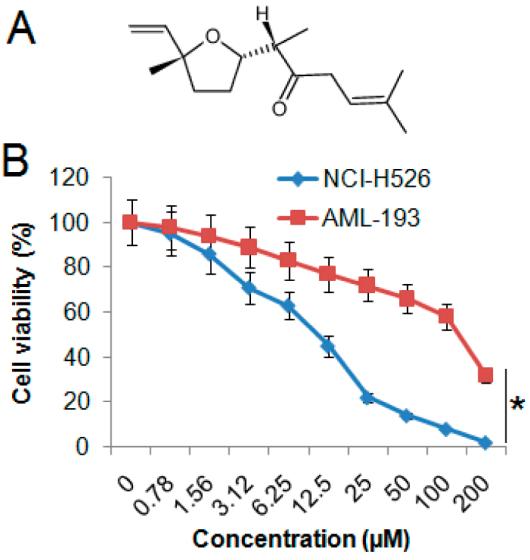

Figure 1. A: Chemical structure of Davanone terpenoid. **B:** Effect of Davanone on cell viability of acute myeloid leukemia cancer cell line NCI-H526 as well as normal AML-193 cell line. Cells were exposed to different concentrations of Davanone molecule for 24 h. Data is shown as means ± standard deviation (*$p<0.05$).

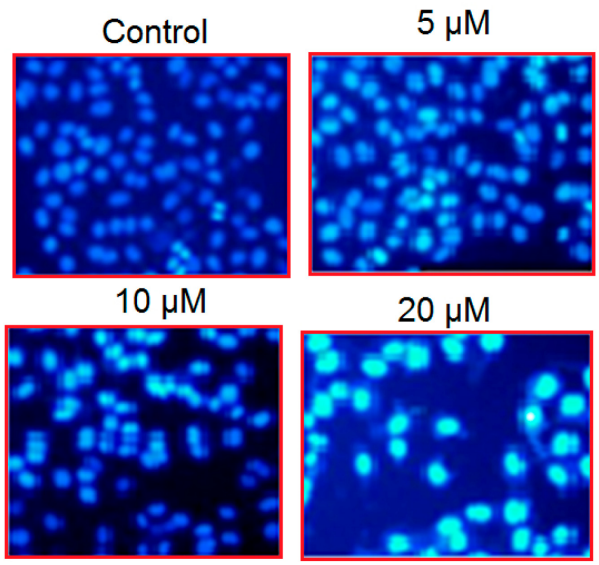

Figure 2. Davanone induced apoptosis in acute myeloid leukemia cancer cell line NCI-H526, evaluated through DAPI staining at various drug concentrations as indicated. The Figure shows dislocated nucleus, damaged DNA and chromatin condensation. The experiments was done in triplicate.

dous ability of the test molecule in bringing about apoptosis. The results revealed that in control, 5, 10 and 20 µM doses the percentage of apoptotic cells was 1.2%, 12.5%, 17.8% and 24.12% respectively (Figure 3), indicating induction of dose-dependent apoptosis by the test molecule. Further, the expression of caspase-3, Bax and Bcl-2 was estimated by western blotting analysis. These proteins are apoptosis-related and their expressions are informative regarding apoptosis. The results showed that the exposure to the test molecule led to upregulation of caspase-3 and Bax expressions, simultaneously downregulating the expression of Bcl-2 (Figure 4).

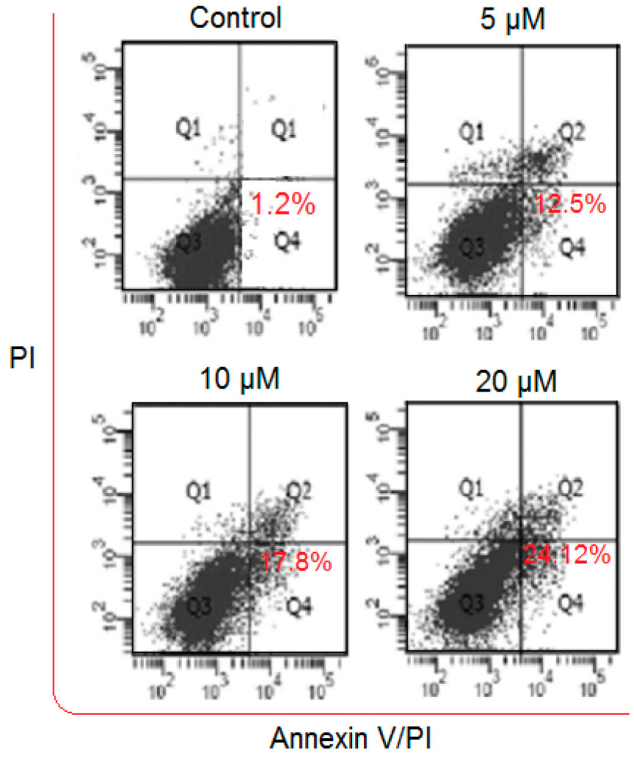

Figure 3. Apoptotic results of NCI-H526 cells induced by 5, 10, 20 µM of Davanone for 48 h, using flow cytometry with Annexin V-FITC/PI binding assay. The Figure shows increasing number of apoptotic NCI-H525 cells on Davanone treatment compared with untreated controls. The experiments were performed in triplicate.

Assessment of MMP and ROS percentage in AML cell line NCI-H526 after Davanone treatment

The effects on MMP and ROS percentage by the test molecule were assessed through flow cytometry in NCI-H526 cells and the results indicated that on increasing doses of the test molecule (control, 5, 10 and 20 µM) MMP percentage decreased sharply from 100% to about 30% (Figure 5A) and ROS percentage increased sharply from 100% to about 250% (Figure 5B). Hence, it is evident that the test molecule targeted both MMP and ROS levels in a dose-dependent manner.

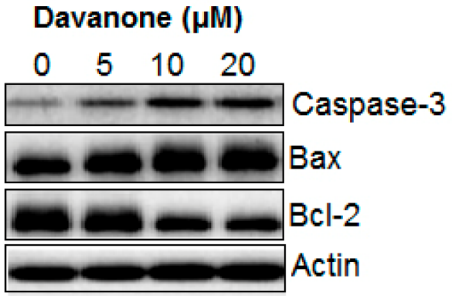

Figure 4. Davanone triggers the stimulation of apoptosis-related proteins in AML cell line NCI-H526. After exposure to test molecule (5, 10, 20 µM) for 12h, cell lysates were prepared and western blot analysis was performed against Bcl-2, Bax and caspase-3. Actin was used as internal control. The Figure shows enhanced Bcl-2 activity compared with untreated controls. The experiments were performed in triplicate.

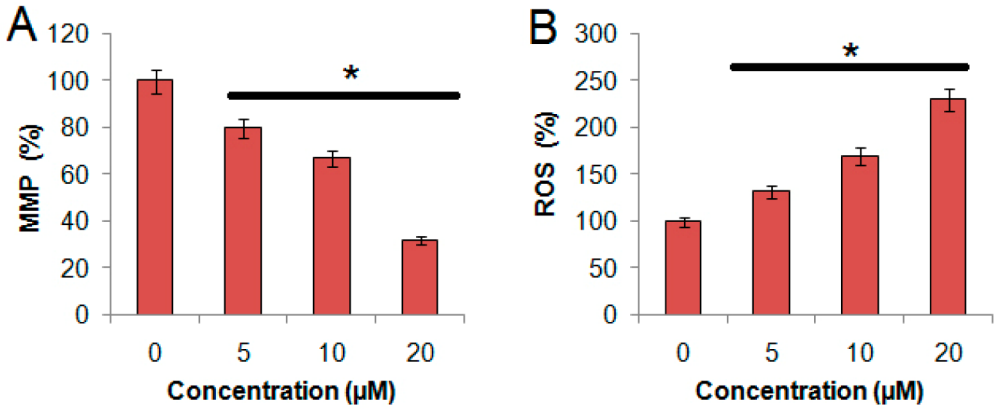

Figure 5. A: Effect of 0, 5, 10 and 20 µM dose of davanone on the mitochondrial membrane potential of acute myeloid leukemia cell line NCI-H526. On increasing the dose concentration a significant decline was observed in MMP. 10^4 cells in each sample were examined through flow cytometry. B: Effect of 0, 5, 10 and 20 µM dose of davanone on the reactive oxygen species (ROS) of AML cell line NCI-H526. Enhanced ROS production in NCI-H526 cells on treatment with Davanone (control, 5, 10, 20 µM) for 24 h was observed. The experiments were performed in triplicate (*p<0.05).

Effects of test molecule on cell migration and invasion of AML cell line NCI-H526

In vitro cell invasion and wound healing assays were performed to assess the invasion and migration ability of NCI-H526 after Davanone treatment, respectively. The results indicated that both the cell invasion and migration were significantly suppressed in a dose-dependent manner. The number of cells migrated was calculated after 24 h of treatment with different concentrations of test molecule and the results clearly indicated that cell migration in negative control cells was not hampered much but in case of treated-cells, migration was immensely suppressed (Figure 6). After treatment of target cells with different concentrations of Davanone (control, 5, 10 and 20 μM) the number of invaded cells was seen to reduce sharply (Figure 7).

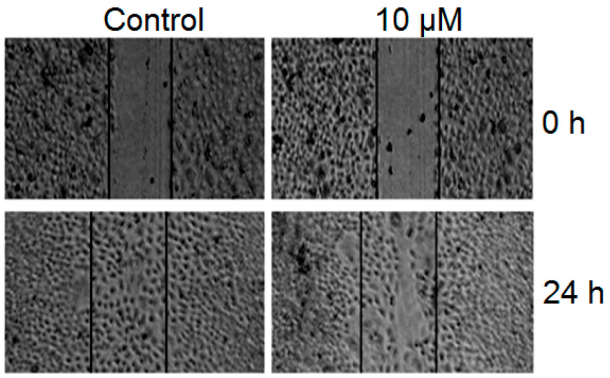

Figure 6. Impact of Davanone on acute myeloid leukemia cell line NCI-H526. Cell migration was measured by wound healing assay. The wound length depicts the number of migrated cells and the Figure shows that the number of migrated cells reduced significantly after Davanone treatment in contrast to the control cells after 24h.

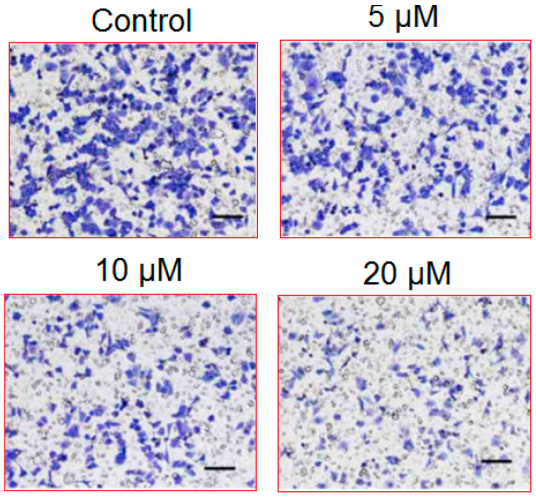

Figure 7. The effects of Davanone terpenoid on cell invasion of acute myeloid leukemia cells were observed through transwell assay. The Figure shows the number of invaded cells in controls and Davanone-treated cells. It is obvious that invaded cells reduced after Davanone exposure. The experiments were performed in triplicate.

Davanone targets PI3K/AKT/MAPK signalling pathway in AML cells

Western blotting analysis was performed in order to assess the expressions of proteins related to PI3K/AKT/MAPK signalling pathway. What was revealed was that the expressions of p-AKT, p-PI3K and p-P38 were significantly delayed and the expressions of AKT, PI3K and P38 were almost constant on increasing the doses of test molecule. This clearly indicates that Davanone results in altering the protein expressions of PI3K/AKT/MAPK signalling pathway.

Discussion

Typically AML is associated with hematologic progenitor or stem cells and is a lethal type of hematological disorder. The main reasons that increase the lethality of this malignancy is the inability to selectively targeting and eradicating the cancerous leukemia stem cells (LSC). LSCs differ from most of the differentiated blast cells with distinctive but unique biological activities, thus in the maximum number of cases these cells are not efficiently targeted through standard chemotherapeutic agents. Stem cells play a fundamental role in relapse, genesis and endurance of AML. In the recent past studies on illustrating the molecular mechanisms that could be selectively targeted for apoptosis induction have been intensified [19]. Such methods include targeting of caspases

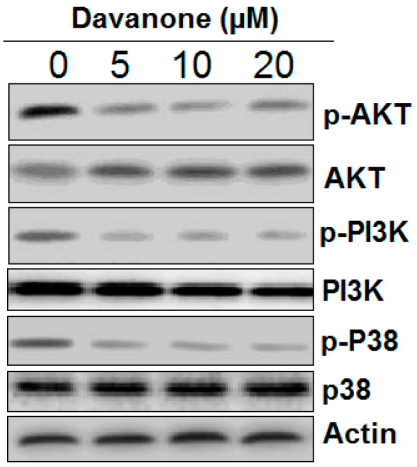

Figure 8. The effects of the test molecule on PI3K/AKT/MAPK signalling pathway in NCI-H526 cells was estimated by western blotting analysis. Treated cells revealed a dose-dependent decrease in the levels of p-PI3K, p-AKT and p-P38. Actin was used as internal control.

[(cysteine-aspartic proteases - a family of protease enzymes that play an important role in automatic cell death (including necroptosis, pyroptosis and apoptosis)], targeting PI3K/AKT/MAPK signalling pathway and mitochondrial membrane potential (MMP). Terpenoids are pharmacologically active compounds that have a potential to curb cancer through different modes of actions including induction of apoptosis. Perillyl alcohol (POH) and D-limonene are monoterpenes and are potent anticancer agents against different human cancers [20]. Herein, the current study was carried out to examine the effects of Davanone terpenoid on cisplatin-resistant AML cell growth by inducing caspase-dependent apoptosis and for that an array of assays were performed. MTT cell viability assay showed that the cell viability in AML cells was delayed significantly as compared to the normal cells in dose-dependent manner. Then apoptosis analysis was performed through DAPI and annexin V/PI staining, which revealed remarkable increase in the number of apoptotic cells and those cells were seen with disrupted, fragmented, reduced and contracted nuclei as compared to negative control cells. It was also seen that the number of apoptotic cells enhanced significantly on increasing the dose of the test molecule, thus apoptosis was observed to be dose-dependent. Western blotting analysis was performed to detect the expression of apoptosis-related proteins and the results revealed that it considerably enhanced the expression of caspase-3 (caspase-dependent apoptosis) and Bax with delaying the Bcl-2 protein expressions. Thereafter, flow cytometry was performed to unveil the effects of the test molecule on ROS and MMP percentage of target cells, with the results depicting that MMP was sharply decreased and ROS production was increased significantly with increased doses of Davanone molecule. Further, wound healing assay and cell invasion assay were performed to assess the effects of Davanone terpenoid on cell migration and invasion of target cells. The results were quite amazing as both the number of invaded as well as migrated cells decreased tremendously with increasing doses of the test molecule. Finally, western blotting analysis was performed to unveil the effects of the test molecule on the PI3K/AKT/MAPK signalling pathway. It was seen that the expressions of p-AKT, p-PI3K and p-P38 were delayed significantly and the expressions of AKT, PI3K and P38 remained constant on increasing the doses of Davanone. All the above said, it is quite evident that Davanone terpenoid can be a promising therapeutic agent but needs more investigations especially its toxicity and *in vivo* studies.

Conclusion

In conclusion, Davanone terpenoid is a potent antileukemic agent that can be used in the systemic therapy of this disease. In the current study it was shown that Davanone exhibits potential antileukemic activity against cisplatin-resistant AML cells through induction of caspase-dependent apoptosis, loss of mitochondrial membrane potential, inhibition of cell migration and invasion and targeting PI3K/AKT/MAPK signalling pathway.

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Conflict of interests

The authors declare no conflict of interests.

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