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Short communication

Anticancer effects of 7,8-dihydromethysticin in human leukemia cells are mediated *via* cell-cycle dysregulation, inhibition of cell migration and invasion and targeting JAK/STAT pathway

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The main focus of this research work was to study the anticancer properties of 7,8-dihydromethysticin against HL-60 leukemia cells. Investigations were also performed to check its impact on the phases of the cell cycle, cell migration and invasion, JAK/STAT signalling pathway and intracellular mitochondrial membrane potential (MMP) and reactive oxygen species (ROS). Cell proliferation was assessed through 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay and effects on colony formation were examined *via* clonogenic assay. Flow cytometry and Western blott analysis were performed to investigate the distribution of cell cycle phases. Flow cytometric analysis was performed for the examination of MMP and ROS production. The effect on JAK/STAT signalling pathway was examined through Western blot analysis. Results depicted that 7,8-dihydromethysticin induced concentration- as well as time-dependent inhibition of cell proliferation in leukemia HL-60 cells. Clonogenic assay indicated potential suppression in leukemia HL-60 cell colonies. The 7,8-dihydromethysticin molecule also caused cell cycle arrest at G2/M-phase along with concentration-dependent inhibition of cyclin B1, D1 and E. ROS and MMP measurements indicated significant ROS enhancement and MMP suppression with increasing 7,8-dihydromethysticin concentrations. Additionally, 7,8-dihydromethysticin led to remarkable dose-reliant inhibition of cell invasion as well as cell migration. Therefore, 7,8-dihydromethysticin should be considered a valuable candidate for leukemia research and chemoprevention.

Keywords: leukemia, 7,8-dihydromethysticin, flow cytometry, cell cycle, signalling pathway

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Leukemia is a life-threatening disorder linked to human bone marrow and blood and is associated with huge mortality and incidences worldwide (1, 2). The development of this malignant disorder takes place in blood-forming tissues. Patients with leukemia show a

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large number of incompletely developed or immature WBCs (3, 4). These immature cells divide to create their copies and resist death caused by cellular damage or age. The frequent division of leukemia cells outnumbers the normal RBCs and erythrocytes, thereby decreasing the oxygen taking capacity of the blood, fighting infection and bleeding control. Pathologically and clinically, leukemia is differentiated into various categories based on the symptoms, history and effected cell types like lymphoblast and myeloid cells (5, 6). Based on this, four different types of leukemia have been identified including acute lymphoblastic leukemia (ALL), acute myeloid leukemia (AML), chronic lymphoblastic leukemia (CLL) and chronic myeloid leukemia (CML) (7, 8). ALL and AML are identified with higher frequency in children and adults, respectively. Conventional chemotherapeutic agents are usually effective against leukemia but the side-effects associated are much more serious (9, 10). The overall survival in leukemia patients post-chemotherapy remains very low due to the high possibility of disease recurrence and deadly side-effects like cardiac events. Thus, there is a pressing need for treatment options for leukemia with reduced side-effects and increased overall survival rates. The HL-60 cell line is a type of human leukemia cells that has been used in laboratory research on physiology and blood formation. These cells proliferate in a suspension culture rich in antibiotic chemicals and nutrients. This cell line is derived from patients with acute promyelocytic leukemia and predominantly exhibit neutrophilic promyelocytic morphology. HL-60 cells are mostly used as a cell model to evaluate pharmacological and physiological effects on myeloid differentiation and other aspects. Natural products are comprised of a huge number of chemicals that pose no threat to naturally balanced systems in humans and also act as potential pharmacological agents (11–15). Naturally occurring Kava plant comprises of different chemical entities including kavalactones. One such kavalactone is dihydromethysticin (DHM), which has been regarded as a remarkable medicinal and pharmacological agent (16). Dihydromethysticin has been reported to exhibit significant activity such as anticancer, anticonvulsant, analgesic and anxiolytic and it acts as a monoamine oxidase B reversible inhibitor and positive $GABA_A$ receptor allosteric modulator (17–20). The current study was designed to unveil the antitumor activity of 7,8-dihydromethysticin in human leukemia HL-60 cells along with examining its effects on cell cycle progression, mitochondrial membrane potential (MMP), reactive oxygen species (ROS), inhibition of cell migration and invasion and JAK/STAT signalling pathway.

EXPERIMENTAL

Cytotoxicity evaluation

Leukemia HL-60 cells were plated onto 96-well plates and harvested at 75 % of confluence. Plated cells were exposed to different concentrations of 7,8-dihydromethysticin *viz.* 0, 10, 50, 75 and 100 μmol L^{-1}. 7,8-Dihydromethysticin (HPLC purity ≥ 98 %) was procured from Shanghai Chemical Reagent Co Ltd, China. The time duration of treatment with the currently tested molecule at 37 °C of incubation varied from 24 and 48 h. Dimethyl sulphoxide (DMSO) vehicle (0.2 %, *V*/*V*) was used as a negative control. Cisplatin (Sigma Aldrich, China) was used as a reference for the positive control. 7,8-Dihydromethysticin treatment was followed by incubation with MTT solution of 0.5 mg mL^{-1} concentration for 4 h and 37 °C. The media was completely removed and the extraction of formazan was

done in DMSO (200 μL) at room temperature for 10 min. Finally, the optical density (OD) was recorded with 570 nm of wavelength and using a microplate reader (BioRad), repeating twice in triplicates.

Colony formation assay

Leukemia HL-60 cells were cultured in 6-well plates using RPMI-1640 medium augmented with 10 % fetal bovine serum at a density of 3×10^3 each well. Cultured cells were treated with 7,8-dihydromethysticin at variant concentrations of 0, 25, 50 and 100 μmol L^{-1}, for one week followed by fixation and staining with 4 % paraformaldehyde and 0.1 % crystal violet respectively (Beyotime, China). Utilizing a digital camera, stained cells were photographed and analysed (Olympus, Japan).

Cell cycle phase assessment via *flow cytometry*

Flow cytometric analysis for cell cycle phase distribution was performed to leukemia HL-60 cells. These cells were seeded at a density of 1×10^5 cells in each well of 6-well plates. Seeding was followed by 7,8-dihydromethysticin treatment at changing concentrations *viz*. 0, 25, 50 and 100 μmol L^{-1} and incubation for 24 h. 7,8-Dihydromethysticin treated cells were then phosphate-buffered saline (PBS) washed and fixed in 80 % ethanol and stored overnight at 4 °C. Afterward, treated cells were again washed with PBS and subjected to 5 μg mL^{-1} RNase and propidium iodide (20 μg mL^{-1} of PI) staining at 37 °C in a dark room for half an hour. Finally, cell cycle analysis was performed with a flow cytometer (FACScan; NJ, USA) and DNA content was collected for cell cycle phase distribution with ten thousand cells per sample.

ROS and MMP analysis by flow cytometry

Leukemia HL-60 cells were subjected to staining with fluorescent dye tetramethylrhodamine methyl ester (TMRM) and analysing with a flow cytometer for MMP assessment. After treatment with different 7,8-dihydromethysticin concentrations (0, 25, 50 and 100 μmol L^{-1}) cells were stained in the dark with TMRM (200 μmol L^{-1}) in PBS for about 15 min at 37 °C. Orange fluorescence intensity was recorded and analysed using flow cytometry. For ROS estimation, leukemia HL-60 cells were harvested from 96-well plates and subjected to 7,8-dihydromethysticin treatment at variant concentrations *viz*. 0, 25, 50 and 100 μmol L^{-1}. Afterward, cells were washed twice with Hanks' Balanced Salt Solution (HBSS) followed by staining with Dihydroethidium staining (DHE) (100 μL) for half an hour at 37 °C. DCF fluorescence with each cell was obtained by capturing, extracting and analysis through Data Viewer version 3.0 and ArrayScan II Data Acquisition (Cellomics).

Determination of cell migration through transwell chambers

Transwell chambers (Corning Costar, USA) were used to carry cell migration analysis of Leukemia HL-60 cells after 7,8-dihydromethysticin treatment. Briefly, upper chambers were filled with media and Leukemia HL-60 cells at a concentration of 2×10^3 cells each upper transwell chamber and only the medium was placed in lower chambers for osmotic pressure maintenance. Afterwards, cells were treated with 7,8-dihydromethysticin at variant concentrations *viz*. 0, 25, 50 and 100 μmol L^{-1} and incubated for 24 h. Non-migrated

cells from upper chambers were discarded using a cotton swab and the fixation of the membrane was performed using methanol. Migrated cells were stained with crystal violet for 15 min, washed thrice with PBS and captured under a microscope followed by washing with 32 % acetic acid. Absorbance was calculated in a plate reader (EnSpire, Perkin Elmer Corporation) at 590 nm.

Determination of cell invasion through transwell chambers with Matrigel

The effect of 7,8-dihydromethysticin on cell invasion tendency of Leukemia HL-60 cells was performed as per the abovementioned procedure except here transwell chambers were covered with Matrigel (BD Pharmingen) and Bovine serum-free medium was used.

Western blot analysis for determination of expressions of JAK/STAT signalling pathway allied proteins

After treatment of leukemia HL-60 cells with 7,8-dihydromethysticin, cells were harvested and lysed using Tris-HCl, sodium dodecyl sulfate (SDS), mercaptoethanol, glycerol lysis buffer. Protein content was then separated using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and was transferred to hydrophilic polyvinylidene fluoride (PVDF) membranes electrophoretically. Afterwards, membranes were treated with the antibodies against JAK-1, JAK-2, STAT-1, STAT-2, p-JAK-1, p-JAK-2, p-STAT-1 and p-STAT-2 following manufacturer's guidelines. At 1:1000 dilution ratio, horseradish peroxidase-linked secondary antibodies were introduced and membranes were incubated for 2 h at room temperature. Membranes were then washed with PBS and visualization of immunoreactive bands was done with ECL-PLUS/Kit. β-actin was used for normalization.

Statistical analysis

The experimental data are presented as mean ± standard deviation (SD). Data analysis was performed by one-way analysis of variance ANOVA and for multiple comparisons, the Student-Newman-Keuls test was used. $p < 0.05$ was taken to show statistically significant differences.

RESULTS AND DISCUSSION

Effect of 7,8-dihydromethysticin treatment on cell viability of leukemia HL-60 cells

Leukemia treatment remains a big challenge for researchers since a higher number of side-effects, disease reoccurrence and lower survival present the key drawbacks of currently accessible treatment modalities. 7,8-Dihydromethysticin has been previously reported of inducing antiproliferative effects against different human cancer cells including colorectal cancer and osteosarcoma (21, 22). It was also reported to exhibit proapoptotic effects and modulated a number of cancer cell survival pathways. Herein, the effects of 7,8-dihydromethysticin (Fig. 1a) treatment on cell proliferation of leukemia HL-60 cells were evaluated. On increasing the concentrations of 7,8-dihydromethysticin *viz*. 0, 10, 50, 75 and 100 μmol L^{-1}, cell viability decreased remarkably after 24 h and 48 h exposure (Fig. 1b). 7,8-Dihydromethysticin exhibited stronger inhibition of cell viability after 48 h time interval as compared

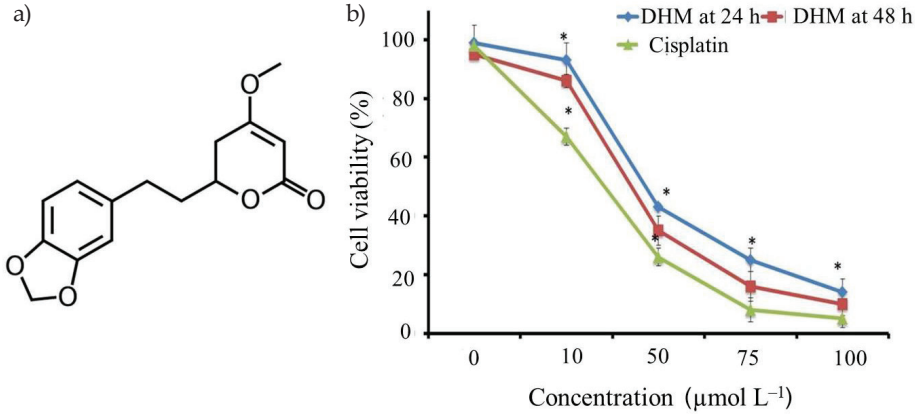

Fig. 1. a) Chemical structure of 7,8-dihydromethysticin; b) MTT viability assay was used for the determination of cell proliferation. Cisplatin (Sigma Aldrich, Mainland, China) was used as a positive control. Results indicated that 7,8-dihydromethysticin treatment in HL-60 cells had induced concentration-dependent cytotoxicity and showed significant results compared to cisplatin. All data are represented as mean ± SD and experiments were repeated three times, $p < 0.05$.

to 24 h, thus showing time-dependent inhibition as well. Further, clonogenic assay revealed significant inhibitory effects on cell colony formation of HL-60 cells induced by 7,8-dihydromethysticin (Fig. 2a). The number of colonies calculated at 0, 25, 50 and 100 μmol L^{-1} was

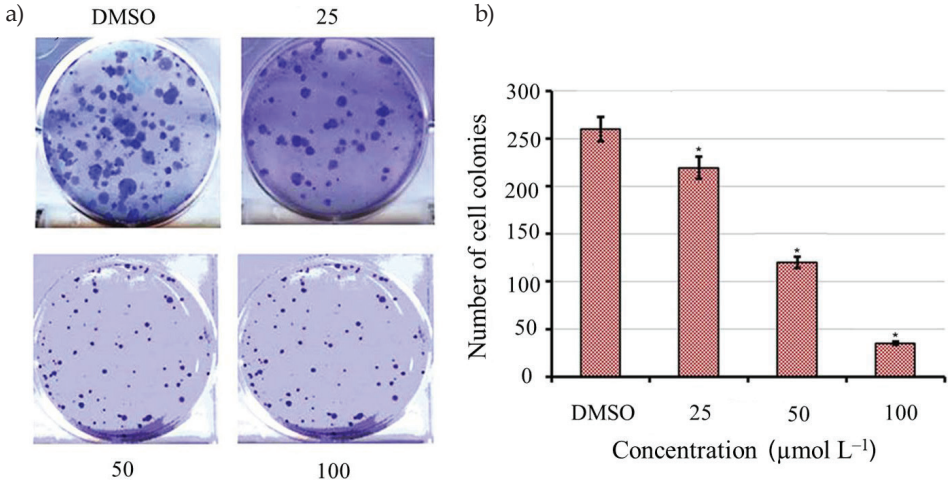

Fig. 2. Clonogenic study of HL-60 cells under a light microscope: a) results showing a decreased number of HL-60 cell colonies; b) the number of HL-60 colonies left at indicated concentrations of 7,8-dihydromethysticin. The data are presented as mean ± SD. *p = 0.05 indicates significant variances from the control group.

260, 225, 125 and 35, respectively (Fig. 2b). Thus, 7,8-dihydromethysticin could induce concentration-dependent inhibition of colony formation in leukemia HL-60 cells.

Effect on cell cycle phase distribution

Further, investigations were carried out to check whether the antiproliferative effects were associated with cell cycle arrest. From the last two decades, various therapeutic targets were recognised and effectively targeted in attaining therapeutic goals. These therapeutic targets include cell cycle arrest, ROS enhancement, inhibitory effects on cell invasion and migration, apoptosis, autophagy, necrosis and different signalling pathways (23–26). Previous studies have reported that 7,8-dihydromethysticin shows significant inhibitory effects on cell cycle progression as shown in the induction of G0/G1-phase cell cycle arrest in MG-63 osteosarcoma cells (21). Herein, flow cytometric analysis was carried out to unveil the impact of 7,8-dihydromethysticin exposure on cell cycle progression in leukemia HL-60 cells. Results revealed a remarkable increase in G2/M-phase cells indicating cell cycle arrest at this phase. After increasing the 7,8-dihydromethysticin concentration *viz*. 0, 25, 50 and 100 μmol L^{-1}, the percentage of G2/M-phase cells started to increase from 14.5, 24.2, 32.1 and 52.3 % respectively (Fig. 3a). Thus, indicating a dose--reliant rise in the number of G2/M-phase cells. Further, Western blot analysis showed dose-dependent inhibition of cyclin-B1, cyclin-D1 and cyclin-E expressions (Fig. 3b). These Western blot results further validated the cell cycle inhibitory effects of 7,8-dihydromethysticin.

Effects on ROS and MMP levels

ROS and MMP play an important role in carcinogenesis as well as its suppression. Enhancement of ROS in cancer cells may cause death or any other adverse effects. MMP decrease allows the release of cytochrome-C by mitochondria into the cytoplasm giving rise to a cascade of reactions that may induce cell death. In most cases, this cell death has

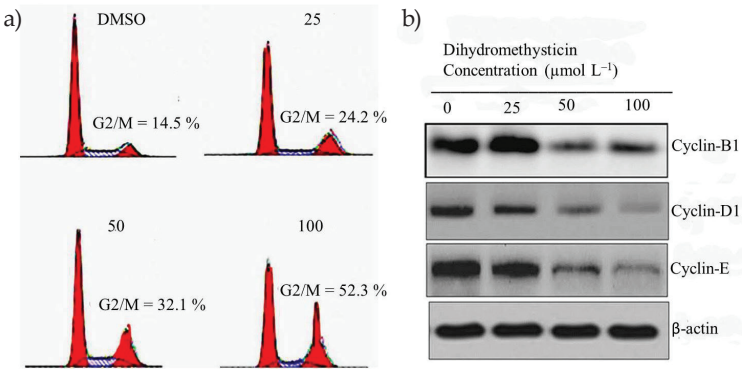

Fig. 3. Cell cycle analysis *via* flow cytometry: a) results indicated that 7,8-dihydromethysticin treatment to HL-60 cells induced G2/M-phase cell cycle arrest; b) Western blot assay revealed expression of cell cycle allied proteins post 7,8-dihydromethysticin treatment. Results showed inhibition of key cell cycle allied proteins (Cyclin-B1, Cyclin-D1 and Cyclin-E) as indicated. All the experiments were done in triplicates.

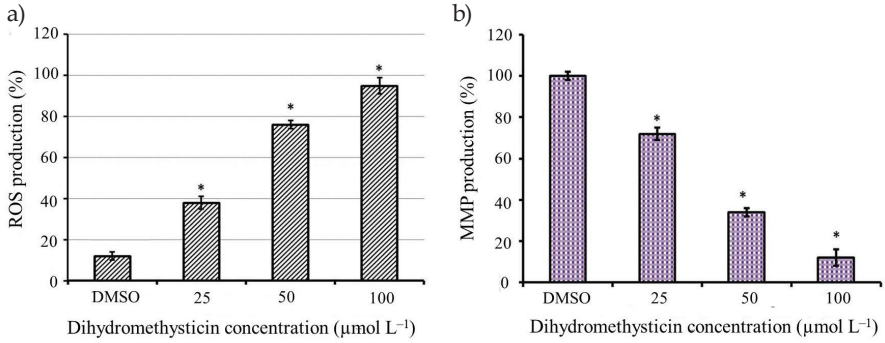

Fig. 4. ROS and MMP assessment in leukemia HL-60 cells after 7,8-dihydromethysticin treatment: a) ROS production in HL-60 cells was observed to increase after treatment with 7,8-dihydromethysticin at indicated concentrations; b) MMP in HL-60 cells was observed to decrease after treatment with 7,8-dihydromethysticin at indicated concentrations. The data are presented as the mean ± SD. * $p < 0.05$ indicates a significant variance from the control group.

been shown to be apoptotic cell death (27, 28). ROS levels were estimated via flow cytometric analysis. Results revealed an incredible increase in the levels of ROS with increasing 7,8-dihydromethysticin concentration. A concentration-dependent increase in the ROS levels was seen as depicted in Fig. 4a. Thus, ROS production increased significantly with increased 7,8-dihydromethysticin concentrations. MMP denotes the functioning of mitochondria and loss in MMP indicates a loss in mitochondrial function. Herein, MMP analysis depicted a remarkable decrease in MMP levels with increasing 7,8-dihydromethysticin concentrations exhibiting dose-dependence (Fig. 4b).

Inhibitory effect of 7,8-dihydromethysticin treatment on cell migration and invasion

The metastatic disease often proves more hazardous than a non-metastatic disease, because of the far dispersal of the disease from the origin. Therefore, cell migration and invasion play a key role in deciding the fate of malignant metastatic cancers. 7,8-Dihydromethysticin was previously reported to inhibit cell migration and invasion in human colorectal cancer cells *via* suppression of NLRC3/PI3K signalling (22). Herein, the cell migration tendency of leukemia HL-60 cells was assessed post-7,8-dihydromethysticin treatment. Results depicted a substantial suppression of cell migration with increased 7,8-dihydromethysticin concentrations (Fig. 5a). Transwell chambers coated with Matrigel were utilized for the estimation of 7,8-dihydromethysticin treatment on cell invasion tendency in leukemia HL-60 cells. It can be seen from the results that the number of invaded cells reduced with the increased 7,8-dihydromethysticin concentrations (Fig. 5b). Thus, both these complementary assays indicate that the tested molecule has substantial potential to inhibit cell invasion as well as cell migration in HL-60 cancer cells.

Effect of 7,8-dihydromethysticin treatment on JAK/STAT signalling pathway

The JAK/STAT pathway (Janus kinase-signal transducer and activator of transcription) is a key signalling pathway that transfers signals to the nucleus from membrane receptors.

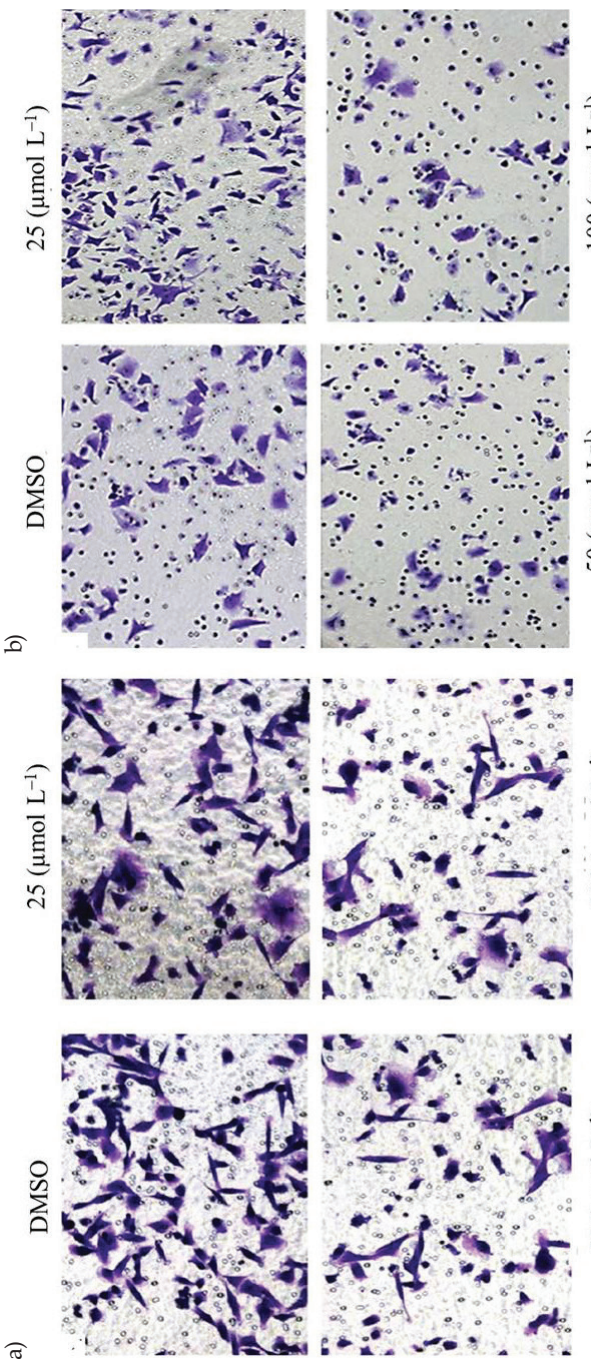

Fig. 5. Cell migration and invasion analysis: a) Cell migration results depicted that 7,8-dihydromethysticin treatment caused suppression of cell migration in HL-60 cells as indicated, b) Cell invasion was analysed *via* transwell chambers coated with Matrigel after 7,8-dihydromethysticin treatment in HL-60 leukemia cells. 7,8-Dihydromethysticin treatment caused a decrease in cell invasive ability of HL-60 cells as indicated. All the data are represented as mean ± SD and experiments were done in triplicates.

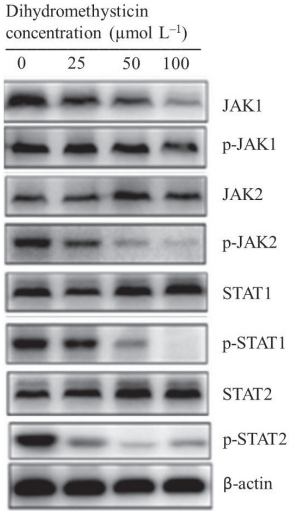

Fig. 6. Western blot analysis for the determination of effects of 7,8-dihydromethysticin treatment on the JAK/STAT signalling pathway in HL-60 leukemia cells. Results indicated inhibition of JAK-1, p-JAK-1, p-JAK-2, p-STAT-1 and p-STAT-2. Actin was used for normalization.

This pathway is associated with essential growth factors and cytokines, regulating a number of cellular events including immune system development and haematopoiesis (29). Herein, the effects of 7,8-dihydromethysticin on the JAK/STAT signalling pathway were evaluated. The Western blot analysis was performed to study the impact of 7,8-dihydromethysticin treatment on JAK/STAT signalling pathway allied proteins. Results revealed that 7,8-dihydromethysticin decreased p-JAK-2, p-STAT-1, p-STAT-2 levels and increased STAT-1, STAT-2 levels (Fig. 6). This indicated 7,8-dihydromethysticin targeted the JAK/STAT signalling pathway hence contributes to its anticancer action.

CONCLUSIONS

Taking together, the abovementioned results indicate that 7,8-dihydromethysticin may be a potent antitumor agent against leukemia, as revealed by its inhibitory effects on cell cycle, cell migration and invasion as well as targeting JAK/STAT signalling pathway and enhancing ROS.

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