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ORIGINAL ARTICLE

Inhibition of human leukemia cells growth by juglone is mediated via autophagy induction, endogenous ROS production, and inhibition of cell migration and invasion

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Summary

Purpose: *The purpose of the study was to examine the anticancer properties of a natural chemical entity - juglone molecule - against human HL-60 promyelocytic leukemia cells along with assessing the effects on normal human monocytes. Juglone molecule was examined for its role in autophagy induction, endogenous ROS production, and inhibition of cell migration and invasion.*

Methods: *Cell viability was evaluated by MTT assay and clonogenic assay was performed to analyse colony formation. Autophagy studies were carried out by transmission electron microscopy (TEM) and western blotting analysis. Mitochondrial morphology was observed through MitoTracker Red CMXRos, and mitochondrial ROS production was assessed through confocal microscopy. The cell invasion and migration was assessed via transwell chambers with and without Matrigel.*

Results: *AMTT assay revealed significant, selective (less cytotoxicity towards normal cells) and dose-dependent inhibition of HL-60 leukemia cells and clonogenic assay showed impressive decrease in the number of colonies on increased doses of the molecule. TEM analysis showed formation of autophagosomes and induction of cellular damage. Western blotting assay indicated a significant increase in LC3-I and LC3-II and a slight increase in Beclin-I. Confocal microscopy revealed tremendous increase in ROS concentrations in a dose-dependent manner. Transwell chamber assay revealed significant, dose- dependent inhibition of cell migration and invasion.*

Conclusion: *Juglone induced anticancer effects on promyelocytic HL-60 leukemia cells mediated via autophagy induction, endogenous ROS production, and inhibition of cell migration and invasion, thus indicating that juglone may be a potential lead molecule against HL-60 promyelocytic leukemia cells.*

Key words: *promyelocytic leukemia, juglone, autophagy, confocal microscopy*

Introduction

Leukemia is a life-threatening disease found in blood and bone marrow. A huge number of deaths and new diagnosed cases of leukemia are registered every year globally and increasing trend [1-3]. Leukemia arises in blood forming tissues with the production of premature white blood cells. Leukemia reduces oxygen carrying efficiency of blood and its other functions too [4-6]. Leukemia is a complex malignancy and categorized as acute or chronic and on the basis of affected cell types [7,8]. Despite the advances achieved in the field of cancer research, the survival rates after treatment of leukemia remain very poor due to heavy life threatening side-effects of chemotherapeutics and disease relapse [9,10]. Different leukemia types are treated with different therapeutic agents like

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cytarabine, daunorubicin and all-trans retinoic acid (ATRA) which are used for the treatment of acute myeloid leukemia. Vincristine is used for acute lymphoblastic leukemia, imatinib for chronic myeloid leukemia and cyclophosphamide, fludarabine, bendamustine and rituximab for chronic lymphoblastic leukemia [11-14]. Using all the above chemotherapeutic agents the survival rate does not change much for leukemia patients, thus desperate need exists for new chemotherapeutics with higher efficiency, lower side-effects, improved survival and no relapse to tackle this deadly malignancy [15,16]. Natural products are a gift from nature with immense pharmacological properties that can be used for treating different cancers [17]. Juglans regia L. (common walnut) belongs to the family of juglandaceae, and comprises many chemical entities with different medicinal implications. Juglone, a primary constituent of walnut leaves, green husks and kernels, induces toxicity and inhibits growth of nearby plants [18,19]. Juglone has also been reported to be useful against many chronic diseases like rheumatisms, heart disease, obesity, diabetes, as well as cancer [20-22]. The current study was undertaken to examine the effects of juglone on leukemia cell growth inhibition mediated via autophagy induction, endogenous ROS production, and inhibition of cell migration and invasion.

Methods

Cell viability assay

Human HL-60 promyelocytic leukemia cells and normal human monocytes (obtained from American Type Culture Collection, ATCC) were cultured in 96-well plates and harvested at 80% confluence. Both leukemia and normal cells after culturing were subjected to treatment with juglone with variant doses (0, 10, 20, 40 and 80 μM) for 24 h at 37°C. Juglone treatment was followed by adding MTT solution (0.5 mg/ml) and cells were incubated at 37°C for 4 h. Afterwards, DMEM medium was decanted and formazan crystals were dissolved in DMSO for 15 min at room temperature. Finally, the optical density was obtained at 570 nm with a microplate reader (BioRad).

Clonogenic assay for estimation of cell colony formation

RPMI-1640 culture medium with 10% fetal bovine serum (FBS) was used to culture human HL-60 promyelocytic leukemia cells in 6-well plates at 3000 cells per well. Harvested human HL-60 promyelocytic leukemia cells were subjected to juglone molecule treatment at different doses (0, 20, 40 and 100 μM). Juglone treatment was followed by fixation in 4% paraformaldehyde and crystal violet staining (Beyotime, China). Finally, a digital camera (model EX-P505, Casio, Tokyo, Japan) was used to capture photographs of colonies and colonies were numbered under light microscope (BX53M, Olympus, Japan).

Transmission electron microscopy for autophagic cell morphology observation

Human HL-60 promyelocytic leukemia cells were exposed to different concentrations of juglone molecule (0, 20, 40 and 100 μM) for about 72 h. Afterwards, pancreatin was used to digest treated cells, followed by fixation at 4°C in 3% glutataldehyde for 2 h. To raise ultrathin sections, cells were first washed in phosphate buffered saline (PBS) and then fixed with osmic acid (1%) for one hour. Thereafter, dehydration of treated cells was performed using acetone and the cells were implanted in epoxide resin. Finally, staining was done with lead citrate and uranyl acetate and investigated under transmission electron microscope (TEM).

Estimation of ROS production

Human HL-60 promyelocytic leukemia cells were treated with juglone for 4 h and ROS were examined via Reactive Oxygen Species Assay Kit. Treated cells were diluted in 10 μM DCFH-DA and incubated with serum-free 1640 medium for about 20 min and at 37°C. Finally, cells were harvested with full confluence and MFI (fluorescence intensity FL-1) was obtained.

Confocal microscopy for determination of mitochondrial morphology

Human HL-60 promyelocytic leukemia cells were treated with changing doses of juglone molecule (0, 20, 40 and 100 μM) for 48 h in serum-free medium, followed by incubation in 100 nM MitoTracker® Red CMXRos at 37°C for about 30 min. Treated HL-60 promyelocytic leukemia cells were then washed in PBS and using a confocal laser scanning microscope IX81-FV1000 (Olympus, Japan) cells were examined for mitochondrial morphology.

Measurements of cell migration and invasion

Measurement of migrated cells was performed in transwell chambers (Corning, Costar, USA). In brief, human HL-60 promyelocytic leukemia cells were treated with changing juglone doses (0, 20, 40 and 100 μM), at 1×10^5 cells per well. The upper chambers were filed with both the culture media and juglone-treated cells and the lower chambers were filled with the medium only to maintain osmotic pressure. After treatment with juglone for 24 h, the membrane was fixed in methanol and the non-migrated cells were taken away with a cotton swab. Crystal violet staining and further washing of treated migrated cells was performed thrice and finally

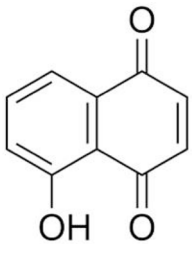

Figure 1. Chemical structure of juglone.

images were captured through microscope. At 590 nm of wavelength the absorbance signal was measured in a plate reader (EnSpire, Perkin Elmer Inc, Waltham, MA, USA). Cell invasion was examined through the above mentioned transwell chambers assay except transwell chambers were coated with Matrigel.

Western blotting assay for determination of expressions of autophagic proteins

For western blotting assay, cell proteins were obtained through protein extraction kit and bicinchoninic acid (BCA) method. Briefly, 20 µg protein from HL-60 promyelocytic leukemia cells were loaded on polyacrylamide gel via SDS-PAGE electrophoresis and then transferred to the Immobilon®-P Transfer Membranes, 0.45µm (PVDF membrane, lot. No: K5PA9282A). Then, appropriate primary antibodies were placed on the membranes overnight at 4°C followed by HRP labelling of appropriate secondary antibodies at 25°C. Finally, bands were analyzed under ImageJ using gel imager (Azure c500, Azure Bio systems, USA).

Statistics

All the data are shown as mean ± standard deviation (SD). Experimental comparisons and analyses were carried out by one-way ANOVA followed by unpaired Student's t-test and taking $p < 0.05$ as statistically significant.

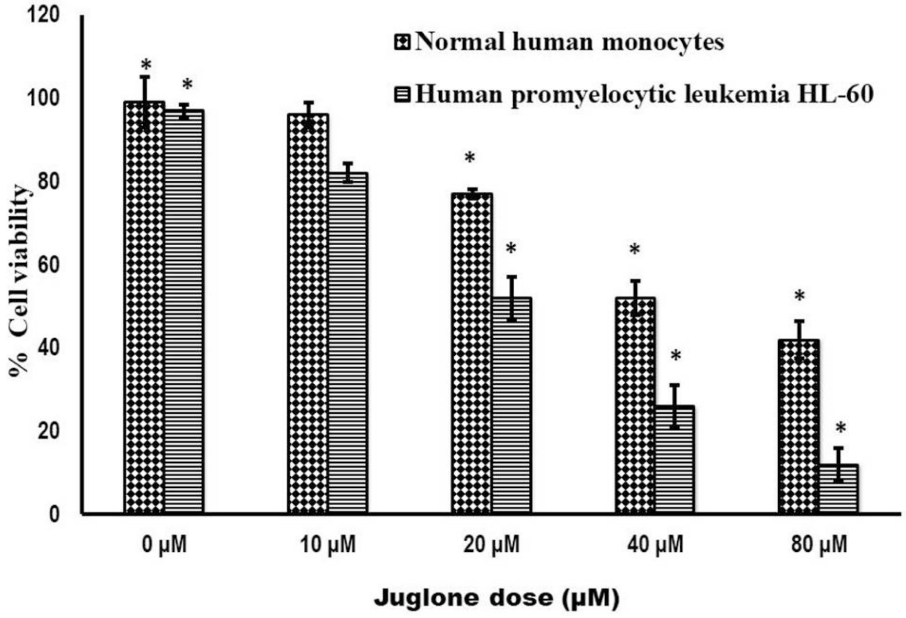

Figure 2. MTT assay for estimation of viability of HL-60 promyelocytic leukemia cells and normal human monocytes after juglone exposure at indicated concentrations. Values are means ±SD of three experiments. Juglone induced selective and dose-dependent cytotoxic effects in the above mentioned cell lines (*p<0.05).

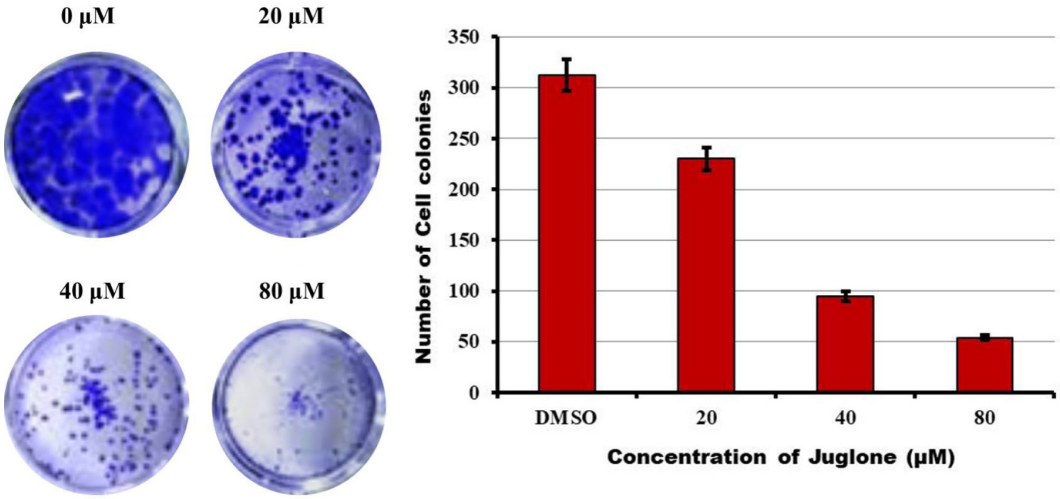

Figure 3. Clonogenic assay revealing the effects of juglone molecule on colony formation of HL-60 promyelocytic leukemia cells. The Figure shows the potential of colony inhibitory effects of juglone in HL-60 cells. Values are means ±SD of three experiments. P< 0.05.

Results

Effects of juglone treatment on cell viability of human HL-60 promyelocytic leukemia cells

Cell growth rate was assessed through MTT cell growth assay. After treatment of human HL-60 promyelocytic leukemia cells and normal monocytes with variant concentrations of juglone molecule (Figure 1) (0, 10, 20, 40 and 100 µM), cell growth rate started to decline at lower molecule doses but on higher doses the growth rate was suppressed significantly to 10% at 100 µM drug dose (Figure 2). The molecule had much less effect of the viability of normal cells. MTT assay proved that juglone induced dose-dependent and selective inhibitory effects on the growth of promyelocytic HL-60 leukemia cells.

Effects of juglone treatment on colony formation of promyelocytic HL-60 leukemia cells

Colony formation was significantly suppressed after treatment of HL-60 promyelocytic leukemia cells with juglone. In control the number of cell colonies was above 300 but on increasing the drug concentration to 20 µM the number of colonies reduced to 230 and with 80 µM juglone the number of colonies was limited to 50 (Figure 3).

Induction of autophagic cell death of HL-60 promyelocytic leukemia cells by juglone exposure

Autophagic studies of HL-60 promyelocytic leukemia cells were carried out by transmission electron microscopy (TEM). Cell growth inhibitory effects of juglone were autophagy-mediated and were validated by TEM, revealing formation of autophagic vesicles/autophagosomes on juglone exposure indicating autophagic cell death. The effects of autophagic intracellular damage were seen increasing on increasing the juglone concentration (Figure 4). Western blotting analysis was performed to analyse the impact of juglone on autophagy-related proteins. The results indicated that the expressions of LC3-I, LC3-II and Beclin-1 all increased on increasing juglone concentrations (Figure 5). Thus collectively TEM and western blotting assays indicated juglone induced autophagic cell death in HL-60 promyelocytic leukemia cells.

Effect on mitochondrial morphology and ROS production after juglone treatment

Mitochondrial morphology was assessed through MitoTracker®Red CMXRos and it was observed that the poor MitoTracker®Red CMXRos staining was possibly due to decreased mitochondrial membrane potential (MMP). Juglone-treated HL-60 promyelocytic leukemia cells appeared with aggregated and blurred staining in contrast with untreated cells which showed disperse and clear mitochondrial morphology. In addition, vacuoles could be seen inside the cytoplasm of treated cells (Figure 6A). Thus Confocal microscopy revealed significant rise in ROS production after juglone treatment in HL-60 cells. In control the ROS percentage was 8%, with 20 µM dose it was 25% and with 80 µM its highest value was observed, which was around 86% (Figure 6B). These observations indicate remarkable and dose-dependent increase in ROS production leading to HL-60 leukemia cell death.

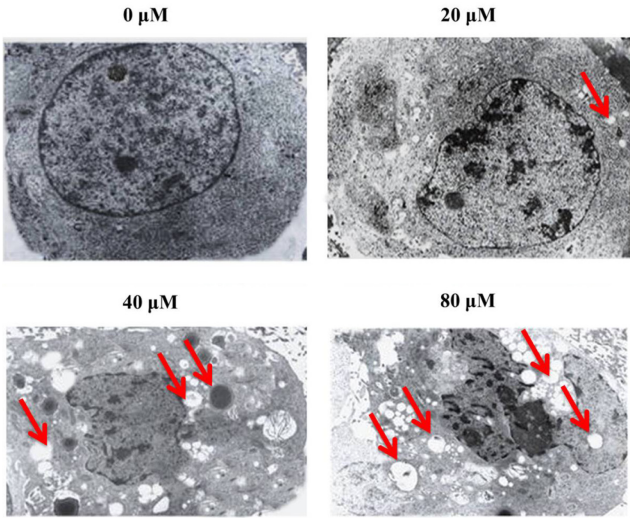

Figure 4. TEM studies for autophagy after treatment of HL-60 promyelocytic leukemia cells with juglone at indicated doses. The Figure shows cellular morphology of HL-60 cells and arrows show autophagosomes. The experiment was done in triplicate.

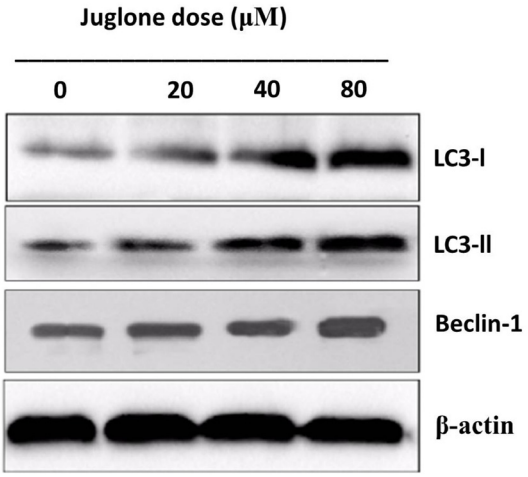

Figure 5. Western blotting study of autophagy-associated proteins (LC3-I, LC3-II and Beclin-I) after juglone exposure at different doses to HL-60 cells. The Figure shows enhanced LC3-I, LC3II and beclin-1 expression level in juglone-treated cells as compared to controls. The experiment was done in triplicate.

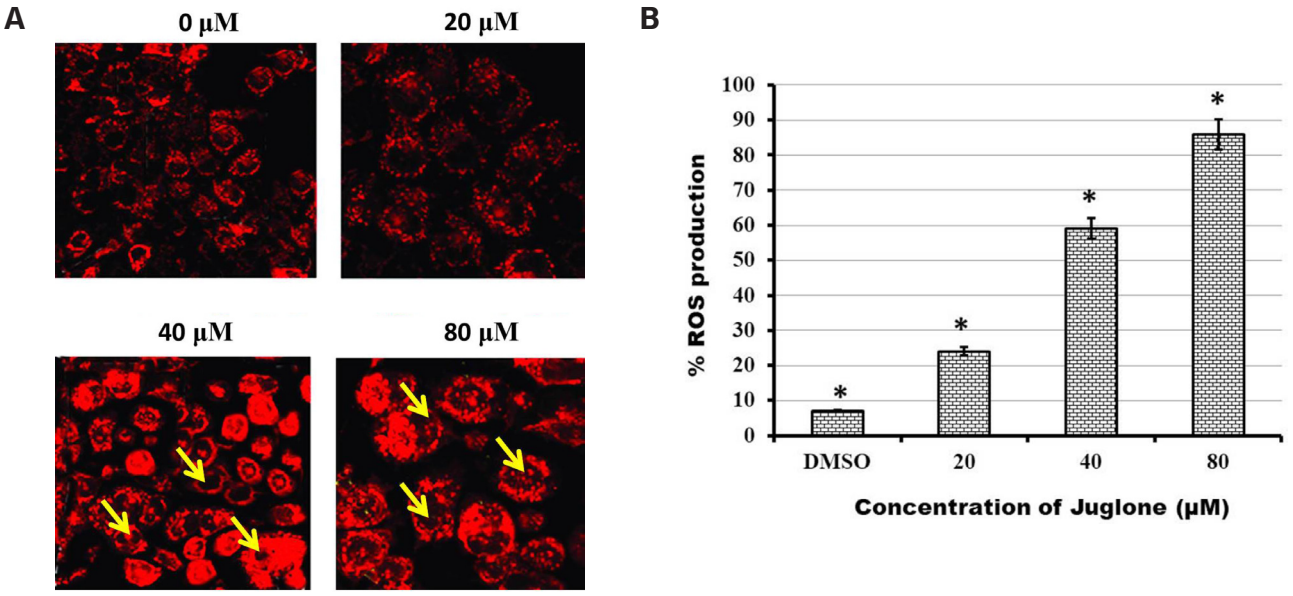

Figure 6. A: MitoTracker®Red CMXRos staining assay for MMP estimation in HL-60 cells after juglone exposure. After 4h of juglone-treated cells the results indicated disrupted mitochondrial morphology (arrows). **B:** Illustrative results of DCFH-DA staining after juglone treatment. The Figure presents quantitative ROS measurements at indicated doses of the molecule in HL-60 cells, revealing enhancement of ROS production. Values are means±SD of 3 experiments. *p<0.05.

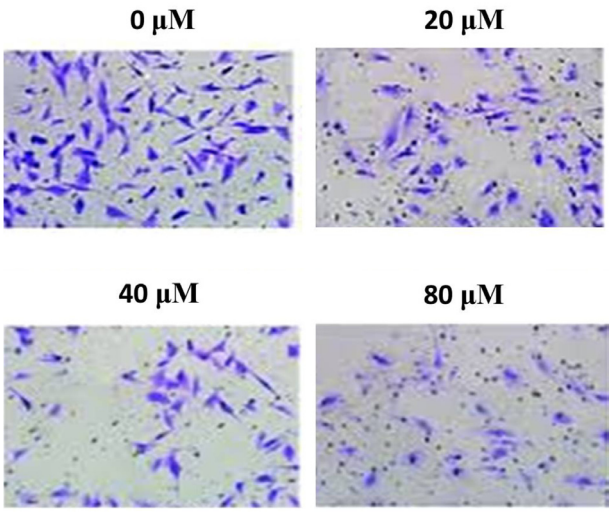

Figure 7. Representative results of transwell assay for cell migration detection in HL-60 cells at different concentrations. The results indicate considerable cell migration inhibition with increasing doses of juglone. The experiment was done in triplicate.

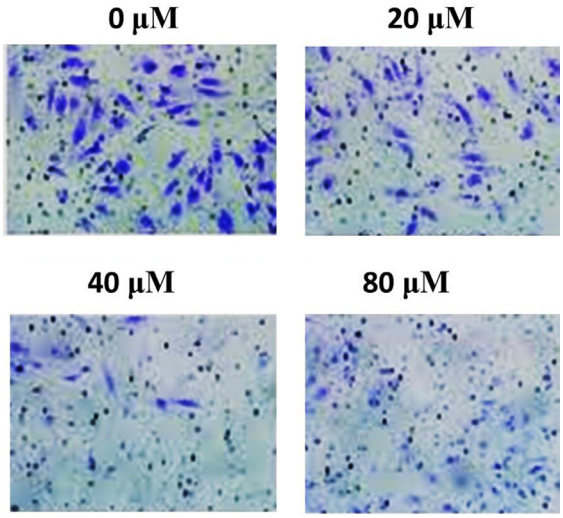

Figure 8. Representative results from transwell chambers coated with Matrigel for estimation of the number of invaded cells after the cells were treated with increasing doses of juglone molecule. The results presented in the Figure show decreased number of invaded cells in drug-treated cells as compared to controls. The experiment was done in triplicate.

Impact of juglone treatment on cell migration and invasion of HL-60 promyelocytic leukemia cells

The effect of juglone treatment on cell migration of HL-60 cells was assessed through transwell chambers assay. After treatment with changing molecule concentrations (0, 20, 40 and 100 µM) the results indicated remarkable decrease in the number of migrated cells (Figure 7). It was observed that when the concentration increased the cell migration inhibition was directly proportional.

The effects on cell invasion potential of HL-60 leukemia cells by juglone was examined through transwell chambers coated with Matrigel. The results revealed that on increasing the doses of juglone molecule the number of invaded cells decreased significantly (Figure 8). Thus considering the results of transwell assay it is evident that juglone treatment of HL-60 cells inhibited both cell migration and invasion in a dose-dependent manner.

Discussion

Leukemia is among the top 10 lethal malignancies threatening to human life. Lack of effective treatments amplify its lethality. Thus to curb this malignancy, new and effective treatment options and approaches are the need of the hour [23,24]. Autophagy is a type II phenomenon of programmed cell death and serves as a therapeutic target in tackling different cancers. Autophagy is hallmarked by the formation of autophagosomes or autophagic vesicles. The current study of juglone molecule was designed to unearth its anticancer potential against human HL-60 promyelocytic leukemia cells which was shown to be mediated via autophagy induction, endogenous ROS production, and inhibition of cell migration and invasion. The effect on the proliferation rate of HL-60 cells by juglone was assessed by MTT viability assay, with the results suggesting incredible dose-dependent inhibitory effects on cell proliferation. Juglone molecule on colony formation was assessed through clonogenic assay, revealing significant inhibition of colony formation dose-dependently. Next, the inhibitory effects on cell growth and proliferation were proved to be autophagy-mediated confirmed through TEM and western blotting analysis. The results showed clearly the dose-dependent enhancement in the rate of autophagic cell death as well as in the expressions of LC3-I, LC3-II and Beclin-1. Further, the mitochondrial morphology and ROS production were evaluated through MitoTracker®Red CMXRos and by confocal microscopy, revealing reduction in MMP through blurred and unclear staining and significant amplification in ROS production responsible for cell death. Thereafter, HL-60 promyelocytic leukemia cells were tested for cell migration and invasion via transwell assay. Cell migration and invasion were observed to reduce on increasing juglone dose, thus revealing anti-migration and anti-invasion effects of juglone molecule on HL-60 promyelocytic leukemia cells.

Conclusion

In conclusion, all the data indicate significant potential of juglone molecule in inhibiting the growth and proliferation of HL-60 promyelocytic leukemia cells. The growth inhibitory effects of juglone were mediated via autophagy induction, endogenous ROS production, and inhibition of cell migration and invasion. Hence the current evaluation of juglone molecule suggests its potential to fight leukemia and can be introduced as lead molecule for leukemia treatment.

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Conflict of interests

The authors declare no conflict of interests.

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