

ORIGINAL ARTICLE

Antitumor effects of helenalin in doxorubicin-resistant leukemia cells are mediated via mitochondrial mediated apoptosis, loss of mitochondrial membrane potential, inhibition of cell migration and invasion and downregulation of PI3-kinase/AKT/m-TOR signalling pathway

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Summary

Purpose: The main purpose of the current study was to investigate the antitumor effects of helenalin - a plant derived sesquiterpene lactone, against doxorubicin-resistant acute myeloid leukemia HL60 cells, along with evaluating its effects on apoptosis induction, mitochondrial membrane potential (MMP), cell migration and inhibition and PI3K/AKT/M-TOR signalling pathway.

Methods: Antiproliferative effects were evaluated with CCK8 cell viability assay and colony formation assay. Cell apoptotic effects were studied by (acridine orange) AO/ethidium bromide (EB) staining assay. To further estimate the extent of apoptosis, flow cytometry using annexin V assay was used. Effects on MMP were estimated by flow cytometry, while transwell migration assay was used to study the effects on cell migration and invasion. Protein expression was estimated by western blot method.

Results: The results showed that helenalin inhibits the

growth of the HL60 cells significantly and exhibited an IC₅₀ of 23.5 μM. In addition, it was observed that the anticancer effects of helenalin are due to induction of mitochondrial-mediated apoptosis which was also associated with enhancement of the expression of Bax and decrease in the expression of Bcl-2. Helenalin also caused loss of MMP in the doxorubicin-resistant HL-60 cells and also inhibited their migratory and invasive properties via modulation of the PI3K/AKT/M-TOR signalling pathway.

Conclusions: In conclusion, the present study reveals that helenalin sesquiterpene lactone exhibits significant antitumor activity in doxorubicin-resistant acute myeloid leukemia HL60 cells by targeting some key pathways and as such this molecule could prove to be a potential drug candidate for future investigations.

Key words: helenalin, acute myeloid leukemia, apoptosis, cell migration, cell invasion

Introduction

Cancer imposes huge health problems throughout the world [1]. The major issues currently are to find novel and efficient chemotherapeutic agents that have minimal side effects. Moreover, there is urgent need to find out anticancer agents that kill cancer cells selectively, without harming normal

cells [2]. Terrestrial plants have been shown to be amazing sources of chemical scaffolds with tremendous medicinal potential [3]. Among the plant-derived natural products sesquiterpene lactones (SQLS) have shown potent pharmacological properties [4]. SQLs include a diverse and large group

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of natural metabolites found across the plant kingdom. Around 500 SQLs have been identified so far from the plant kingdom [5]. In the last couple of decades SQLs have gained considerable attention owing to their potent bioactivities such as anticancer and antimicrobial [6,7]. A number of SQLs have been reported to exhibit significant anticancer activity and many of the SQLs are currently undergoing clinical trials [8]. Helenalin is an important SQL having significant pharmacological potential, with its bioactivities ranging from antimicrobial to anticancer [9,10]. It has been reported to cause growth inhibition of cancer cells through multiple mechanisms [10]. However, the anticancer effects of Helenalin have not been explored against doxorubicin-resistant leukemia cells. This study was therefore undertaken to explore the anticancer effects of helenalin against the doxorubicin-resistant HL-60 human leukemia cells.

Leukemia is one of the destructive malignancies and as per the American Cancer Society estimates around 0.315 million people develop any one form of leukemia every year in U.S. and out of these approximately 0.215 million die of this dreadful disease [11,12]. Although accounting for less than 3% of all malignancies, leukemia is still one of the leading causes of death due to cancer in children and persons below the age of 40 years [13]. Late diagnosis and the lack of potent and safe chemotherapeutic drugs form an obstacle in the treatment of leukemia [14].

The purpose of the present study was to examine the anticancer effects of helenalin sesquiterpene lactone on HL-60 human leukemia cell line along with the evaluation of its effects in mitochondrial-dependent apoptosis, cell migration and invasion and PI3K/AKT/mTOR signalling pathway.

Methods

Cell counting kit-8 (CCK-8) assay

The doxorubicin-resistant HL-60 leukemia cells were inoculated in 96-well plate and subjected to treatment with Helenalin at various concentrations and the number of HL-60 cells was measured at each concentration. The procedures were as follows: the culture Dulbecco's Modified Eagle Medium (DMEM) was discarded and 100 μ L of CCK-8 reagents were added (Beyotime Institute of Biotechnology, Shanghai, China) to a fresh medium. The 96-well plate was incubated in an incubator for 2 h. The optical density (OD) values were measured by a microplate reader at 450 nm wavelength. The cell proliferation rate (%) was calculated as follows: $(\text{OD value of experimental well} - \text{OD value of control well}) / \text{OD value of control well} \times 100$. The colony formation assay was carried out as described previously [15].

AO/EB staining for apoptosis

The HL-60 leukemia cells (0.6×10^6) were grown in 6-well plates. Following an incubation period of around 12 h, the HL-60 cells were subjected to helenalin treatment for 24 h at 37°C. As the cells were discarded, 25 μ L cell cultures were put onto glass slides and subjected to staining with AO and EB (1 μ L). The slides were cover-slipped and examined with a fluorescent microscope. Annexin V/propidium iodide (PI) staining was performed as described previously [16].

Cell migration and Invasion assay

The migration and invasion properties of the HL-60 cells were examined by transwell chamber assay. Briefly, 1×10^4 HL-60 cells were kept in the upper chamber having transwells of 8 μ M pores. In the lower chamber, RPMI medium was placed and this was followed by 24-h incubation at 37°C. Extracellular matrix gel was used in case of cell invasion assay. The non-migrated and non-invaded cells were removed by swabbing. The cells that invaded and migrated to the lower chamber were fixed and stained with crystal violet and finally observed under microscope.

Determination of MMP levels

For determination of the MMP levels, the HL-60 cells were treated with 0, 7.5, 15 and 30 μ M concentrations of helenalin for 24 h and then the MMP in the HL-60 cells was determined as described previously [16].

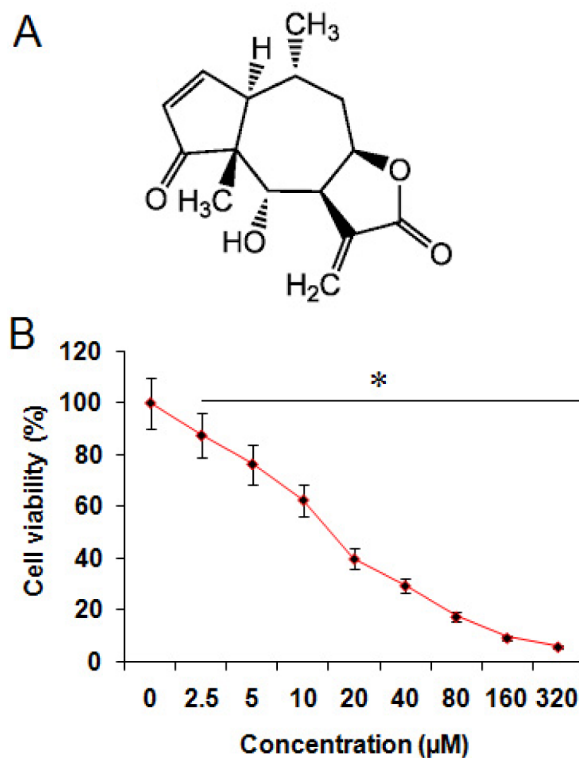


Figure 1. A: Chemical structure of helenalin. **B:** Effect of Helenalin on the viability of the HL-60 cells as determined by CCK8 assay. The experiments were performed in triplicate and presented as mean \pm SD (* $p < 0.01$).

Western blot analysis

Protein expression estimation was carried out by western blotting. The helenalin-treated cells were harvested with centrifugation. The cells were then lysed in lysis buffer containing protease inhibitor. Around 45 μ g of proteins from each sample were subjected to

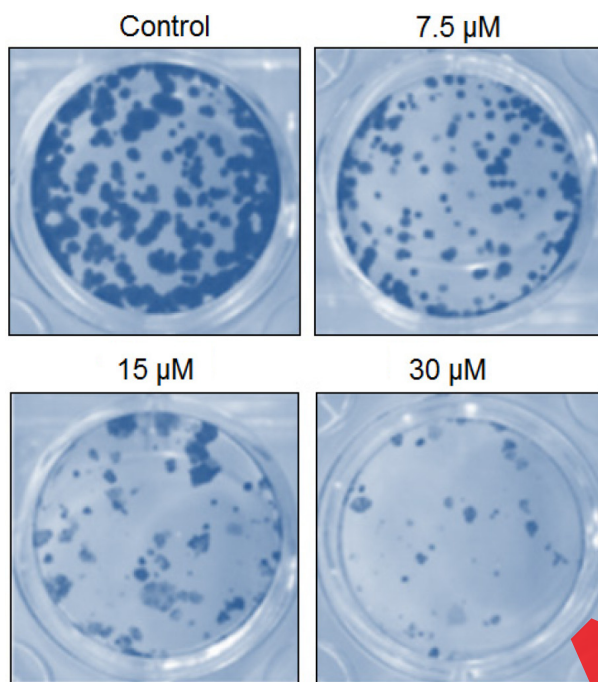


Figure 2. Effect of helenalin on the colony formation of the HL-60 cells as determined by colony formation assay. The Figure shows that helenalin treatment leads to reduction of cancer cell colonies in a dose-dependent manner. The experiments were performed in triplicate.

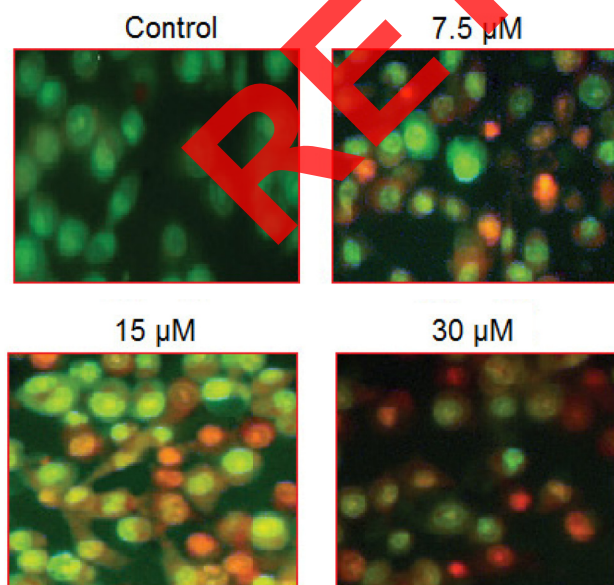


Figure 3. AO/EB assay showing induction of apoptosis in HL-60 at indicated concentrations of helenalin. The results show that the percentage of the orange colored cells increased, indicative of apoptosis of HL-60 leukemia cells. The experiments were performed in triplicate.

separation, followed by transferring to polyvinylidene difluoride (PVDF) membrane. Next, fat-free milk was used to block the membrane at room temperature for 1 h. Afterwards, the membranes were treated with primary antibodies at 4°C overnight. Subsequently, the membranes were incubated with secondary antibodies. Finally, the signal was detected by Odyssey Infrared Imaging System (LI-COR, USA). Actin was used as control for normalization.

Statistics

SPSS statistical software was used for statistical analyses. All the data are shown as mean \pm SD of 3 independent experiments. One way ANOVA and Bonferroni/Dunnet *post hoc* were used for multiple comparisons. $P < 0.05$ was considered statistically significant.

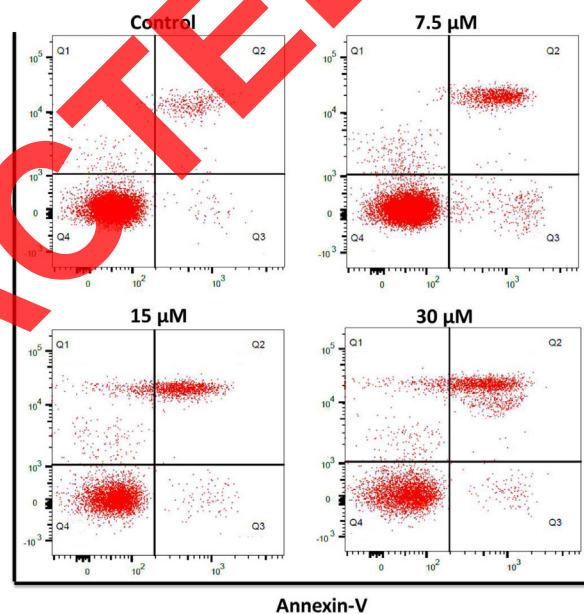


Figure 4. Annexin V/PI assay showing percentage of apoptotic HL-60 cells at indicated concentrations of helenalin. The percentage of apoptotic leukemia cells increased in a concentration-dependent manner.

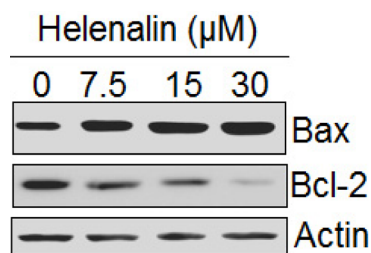


Figure 5. Effect of helenalin on the expression of apoptosis related proteins as depicted by western blot analysis. The results showed that the expression of Bax increased, while the expression of Bcl-2 decreased in a concentration-dependent manner. The experiments were performed in triplicate.

Results

Helenalin inhibits the growth of leukemia cells

The CCK8 assay was employed to investigate the effects of helenalin (Figure 1A) on the proliferation of the leukemia HL-60, which showed this molecule exerts antiproliferative effects on the leukemia HL-60 cells. The IC₅₀ was 15 μM. In addition, the anticancer effects of Helenalin on the leukemia cells were concentration-dependent (Figure 1B). The antiproliferative effects of helenalin were also examined with colony formation assay of the HL-60 cells. The results revealed that helenalin

suppresses the colony formation of the HL-60 cells in a dose-dependent manner (Figure 2).

Helenalin induces apoptosis in leukemia cells

To ascertain if helenalin exerts antiproliferative effects on the HL-60 cells via induction of apoptosis, we carried out AO/EB and annexin V/PI staining. The results showed that the percentage of the orange color cells increased, indicative of apoptosis in the HL-60 leukemia cells (Figure 3). The annexin V/PI staining showed that the leukemia cell percentage increased in a concentration-dependent manner. The apoptotic percentage in-

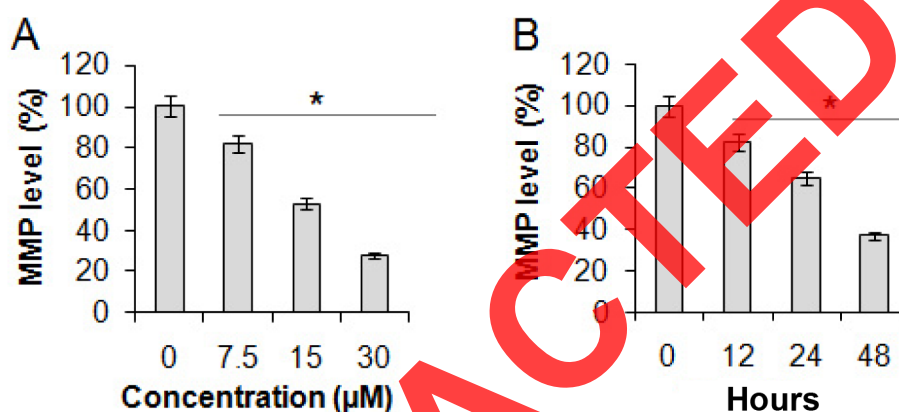


Figure 6. Effect of helenalin on **A:** Mitochondrial membrane potential (MMP) levels at indicated concentrations and **B:** MMP at different time intervals. The results indicated that helenalin led to reduction of MMP in a dose-dependent as well as time-dependent manner. The experiments were performed in triplicate and presented as mean ± SD (*p<0.01).

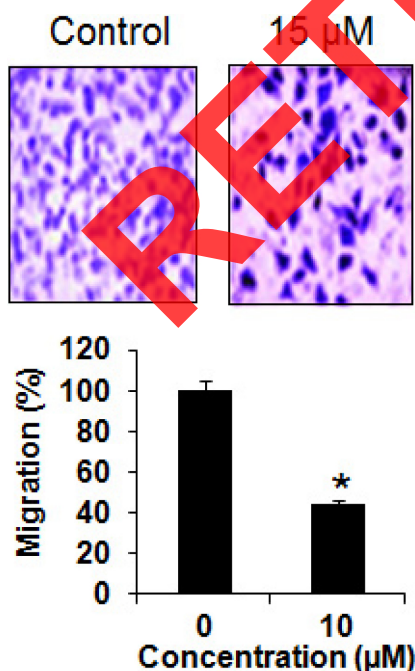


Figure 7. Effect of helenalin on the migration of the HL-60 cells as determined by Transwell assay. The results showed a significant decrease in cell migration at 15µM dose of helenalin. The experiments were performed in triplicate and presented as mean ± SD (*p< 0.01).

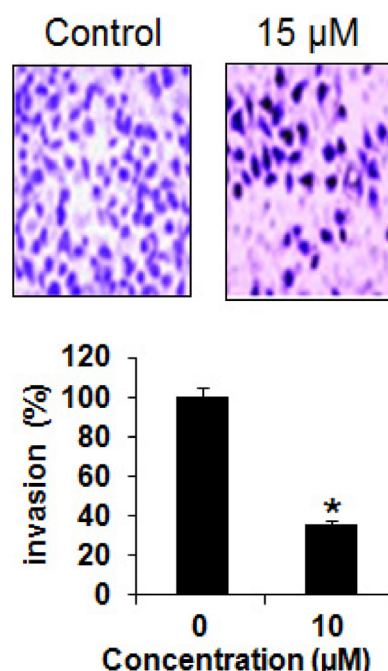


Figure 8. Effect of helenalin on the invasion of the HL-60 cells as determined by Transwell assay. The results showed a significant decrease in cell invasion at 15µM dose of helenalin. The experiments were performed in triplicate and presented as mean ± SD (*p< 0.01).

creased to 25% at 30 μM as compared to the 0.01% in the control (Figure 4). For the validation of apoptosis, the expression of apoptosis-associated proteins was examined and it was found that helenalin caused upsurge of Bax and downregulation of Bcl-2 in HL-60 cells (Figure 5).

Helenalin decreases the MMP levels in the HL-60 cells

The effect of the helenalin was also examined on the MMP levels of HL-60 cells at 0, 7.5, 15 and 30 μM concentrations. The results showed that the levels of the MMP decreased in a concentration-dependent manner (Figure 6A). The effects of helenalin were also examined at IC_{50} at 0, 12, 24, 48 and 96 h time intervals and it was shown that helenalin decreased the MMP levels in a time-dependent manner as well (Figure 6B).

Helenalin inhibits the migration and invasion of HL-60 leukemia cells

The effects of helenalin on the migration and invasion of HL-60 cells were monitored by transwell chamber assay. It was found that helenalin treatment could considerably inhibit the migration of cancer cells in a dose-dependent manner (Figure 7). Similar effects were also found on the invasion of the HL-60 cells (Figure 8).

Helenalin inhibits the PI3K/AKT/mTOR pathway of HL-60 cells

The effects of helenalin were also examined on the PI3K/AKT/mTOR signalling pathway and the results showed that this molecule inhibited the phosphorylation of PI3K, AKT and mTOR proteins as indicated by western blotting (Figure 9). These effects of helenalin were found to be concentration-dependent.

Discussion

Accounting for approximately 3% of all malignancies reported across the globe, leukemia is responsible for significant morbidity and mortality. The chemotherapeutic agents used for the treatment of leukemia exhibit severe adverse effects on the overall health of the patients [17]. Owing to this, there has been increased pursuit to identify and characterize molecules that could be used as lead molecules for the treatment of different types of cancers including leukemia [18]. Helenalin is an important SQL that has been reported to halt the growth of cancer cells [10] and has also been reported to induce autophagy in cancer cells [19]. In yet another study it has been reported to induce apoptosis of renal cancer cells [20]. The anticancer

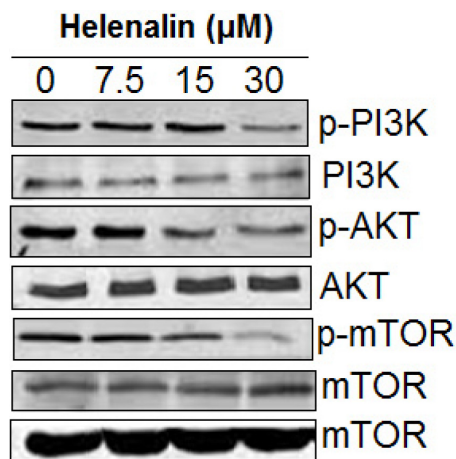


Figure 9. Effect of helenalin on the PI3K/AKT/mTOR signalling pathway as depicted by western blotting at indicated concentrations. Helenalin inhibited the phosphorylation of PI3K, AKT and mTOR proteins. The experiments were performed in triplicate.

activity of helenalin has also been attributed to its potential to inhibit the activity of telomerase [21]. Herein, the anticancer effect of the SQL helenalin was examined against the H-L60 leukemia cells and the results showed that this molecule suppressed the growth of the HL-60 leukemia cells concentration-dependently. The colony formation assay also showed the potential of helenalin to suppress the colony formation. These results are supported by a study wherein nano-encapsulated helenalin has been reported to inhibit the proliferation of breast cancer cells [22]. Apoptosis is an imperative mechanism to eliminate defective cells from the body [23] and herein we found that helenalin could reduce the MMP of H-60 cells which was also associated with induction of apoptosis. Bax and Bcl-2 are the important marker proteins for the induction of apoptosis [24]. In this study we found that helenalin could reduce the expression of Bcl-2 and increase the expression of Bax. The prerequisite for metastasis of cancer cells is their ability to migrate or invade the distant organs of the body [25]. Herein the invasion and migration assays showed that helenalin could suppress both invasion and migration of HL-60 cells. The PI3K/AKT/mTOR signalling pathway has been shown to be deregulated in cancer cells [26] and herein it was found that helenalin could block this pathway, indicative of its potent anticancer effects.

Conclusion

The findings of this study showed that helenalin inhibits the growth of doxorubicin-resistant leukemia cells via induction of mitochondrial

apoptosis. Helenalin could also suppress the migration and invasion of HL-60 cells by targeting PI3K/AKT/mTOR pathway and may therefore prove essential in the innovation of the systemic therapy of leukemia.

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Conflict of interests

The authors declare no conflict of interests.

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