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# Isoalantolactone exerts anticancer effects on human HEC-1-B endometrial cancer cells *via* induction of ROS mediated apoptosis and inhibition of MEK/ERK signalling pathway

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**Isoalantolactone has been shown to inhibit the growth of different cancer cells. The objective of the present study was to evaluate the effects of isoalantolactone on the proliferation of endometrial cancer HEC-1-B cells. Results showed that isoalantolactone suppressed the proliferation of HEC-1-B cells in a concentration-dependent manner and exhibited an $IC_{50}$ of 10 μM. The cytotoxic effects of isoalantolactone were relatively lower against the normal THESC cells. Mechanistic studies revealed apoptosis to be responsible for the isoalantolactone mediated antiproliferative effects. Annexin V/PI assay showed that the percentage of the apoptotic HEC-1B cells increased from 3.74% in untreated cells to 28.9% at 20 μM isoalantolactone. The expression of Bax was significantly increased and that of Bcl-2 was decreased in isoalantolactone treated HEC-1B cells. Analysis of ROS levels revealed that the ROS levels of HEC-1B cells increased with the increase in concentration of isoalantolactone. The ROS levels increased to 210% of the control at 20 μM isoalantolactone. The wound healing and the transwell assays showed that migration and invasion of the HEC-1B cells was significantly decreased upon isoalantolactone treatment. Finally, the effects of isoalantolactone were also evaluated on the MEK/ERK signalling pathway. It was found that isoalantolactone could concentration-dependently block the expression of p-MEK and p-ERK. Taken together, the results suggest that isoalantolactone could prove to be a lead molecule in the development of chemotherapy for endometrial cancer.**

**Keywords**: endometrial cancer, isoalantolactone, apoptosis, invasion, migration MEK/ERK signalling

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**Abbreviations**: AO/EB, acridine orange/ethidium bromide; DCFH-DA, dichloro-dihydro-fluorescein diacetate; HEC-1-B, endometrial cancer cells; PBS, phosphate buffered saline

## INTRODUCTION

Endometrial cancer is one of the lethal gynecological cancers (Kommoss *et al.*, 2018). Unlike other cancers, the incidence of endometrial cancer is increasing at an alarming rate in the United States. Over 90% of patients of endometrial cancer fall in peri-menopausal stage of which 25% females are pre-menopausal (Landrum *et al.*, 2013). Nevertheless, 4% of the younger females with endometrial cancer fall in the age group of 40 years or less, out of which 70% are nulliparous. The most of endometrial patients are diagnosed at stage I of the disease and exhibit 95% 5-year survival rates (Trojano *et al.*, 2019). Clinically and pathologically, endometrial cancer has been categorized into two subtypes: Type I and Type II (Guinde *et al.*, 2018). Type I shares some similarity to endometrioid endometrial carcinoma characterized by genetic predisposition including Lynch syndrome, polycystic ovarian syndrome, obesity, hyper estrogenic state as a result of uneven menstruation and anovulatory cycle (Bogani *et al.*, 2016). Type II is the lethal form of endometrial cancer presenting no similarity to endometrium and is often found in old-aged females. Type II is a high-grade stage endometrial cancer associated with extremely poor prognosis and includes different subtypes like undifferentiated, clear cell and serous cell (Felix *et al.*, 2010). Despite the progresses made in the field of oncology, we still lack potential therapeutics which could completely eliminate this lethal disorder. Nature has served as a rich and diverse source of chemical scaffolds with pharmacological potential (Khursheed & Jain, 2020]. Due to extraordinary structural varieties and synthetic drug analogs, natural products have aided drug design and development. Sesquiterpene lactones are a group of natural products with remarkable pharmaceutical properties (Li *et al.*, 2020; Matos *et al.*, 2020). These compounds are often included in traditional medicine for treatment of serious disorders like inflammation, microbial infections and cancer (Aliarab *et al.*, 2018). Sesquiterpenes are currently in clinical trials for cancer chemotherapy including thapsigargin, artemisinin, parthenolide and several other synthetic analogs (Ghantous *et al.*, 2010). These sesquiterpene lactone-based drugs have been evaluated and found to have anticancer effects against cancer stem cells, as well as target growth and differentiation. Isoalantolactone is one of the pharmaceutically active sesquiterpene lactones (He *et al.*, 2017). Broad spectrum of medicinal activities has been reported for isoalantolactone including antitrypanosomal, anti-inflammatory, antimicrobial, anthelmintic and antifungal activities (Rasul *et al.*, 2013). Isoalantolactone has been found to have strong antibacterial activity against pneumonia causing *Staphylococcus aureus* (Gierlikowska *et al.*, 2020). More importantly, isoalantolactone has been found to suppress the growth of wide range of human cancer cells including leukemia, lung, prostate, ovary, melanoma and colon (Rasul *et al.*, 2013). It has been revealed to promote oxidative stress, apoptosis and also target several signalling pathways (Chen *et al.*, 2018; Khan *et al.*, 2012). Yet no study evaluated the anticancer effects of isoalantolactone against endometrial cancer. Against this background, the present study was designed to evaluate the antiproliferative effects of isoalantolactone against the HEC-1-B endome-

trial cancer cells and to explore the underlying molecular mechanisms.

## MATERIALS AND METHODS

### Chemicals and reagents

Isoalantolactone (98% of purity by HPLC), was procured from the Tauto Biotech Co., Ltd. (Shanghai, People's Republic of China). Unless otherwise mentioned, entire chemicals involved in this research were bought from Sigma-Aldrich (St. Louis, MO, United States). Annexin V/PI, RMPI-1640 culture medium, penicillin, streptomycin, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), acridine orange/ethidium bromide (AO/EB), phosphate buffered saline (PBS), dichloro-dihydro-fluorescein diacetate (DCFH-DA), RIPA lysis buffer, dimethyl sulphoxide, crystal violet and fetal bovine serum (10%, FBS) were bought from Sigma-Aldrich, Beijing, People's Republic of China.

### Cell culture

The endometrial cancer cells HEC-1-B and normal THESC cells were obtained from American Type Culture Collection, Manassas, VA, United States. Culturing of the cell lines was performed in RMPI-1640 medium containing antibiotics (penicillin (100 U/mL) and streptomycin (100 μg/mL) and 10% fetal bovine serum. This mixture was placed under humid conditions within a 5% $CO_2$. The isoalantolactone was dissolved in DMSO (final concentration <1%) and DMSO alone was used to treat control cells.

### Proliferation assay

The antiproliferative effects of isoalantolactone were evaluated against the HEC-1-B cell and THESC cells via MTT assay. In brief, 96-well plates were utilized in seeding of HEC-1-B and THESC cells with a density of $1.5\times10^4$ cells per well. Above 70% of confluence, cells were exposed to isoalantolactone at various doses (0–100 μM) followed by incubation of 48 h at 37°C. Subsequently, 2.5 mg/ml of MTT was added to each well and subjected to additional incubation of 4h under above conditions. Thereafter, the whole cultural media was removed, and formazan crystals were dissolved using 100 μL DMSO. Finally, a spectrophotometer (BD Biosciences, San Jose, CA, United States) was utilized to determine absorbance at 570 nm.

### AO/EB staining, DAPI staining and Annexin V/PI-FITC assay

For apoptotic investigations, the HEC-1-B cancer cells were placed with a density of $0.7\times10^5$ cells/well onto 6-well plates and incubated for 12 h at 37°C. Subsequently, HEC-1-B cells were exposed to isoalantolactone for 24 h at different concentrations viz 0, 5, 10 and 20 μM. Then, treated cells were plated over glass slides. Control cells received only DMSO treatment. In case of AO/EB staining assay, cells treated over glass slides were stained with AO (100 μg/mL)/EB (100 μg/mL) staining solution and then washed once with PBS. Following washing, slides were covered with coverslips and loaded to a fluorescence microscope (BioTek Instruments, Inc., Winooski, VT, United States) for apoptosis analysis.

In case of DAPI staining assay, treated cells were stained using DAPI solution and then covered with coverslips. These slides were investigated for apoptosis under a fluorescence microscope (BioTek Instruments, Inc., Winooski, VT, United States).

In case of Annexin V/PI assay, a similar procedure to that of AO/EB staining and DAPI staining was used except that Annexin V/PI staining was used for quantitative analysis of apoptosis through flow cytometry.

### DCFH-DA staining

The HEC-1-B cells were exposed to isoalantolactone at various concentrations of 0–40 μM within 6-well plates containing $1.5\times10^4$ cells/well. The treatment lasted for 24 h, followed by washing of cells in RMPI-1640 medium and subsequent staining with DCFH-DA (10 μM) at 37°C for 20 min in the absence of light. Cells were rewashed in medium followed by flow cytometry analysis (FACSCalibur, BD Biosciences, San Diego, CA, United States). Isoalantolactone treated DCFH-DA stained cells were pictured under a fluorescence microscope.

### Wound healing assay

Wound healing assay was used to analyze the migratory potential of HEC-1-B cells before and after isoalantolactone treatment. In brief, HEC-1-B cells were placed with a concentration of $1.2\times10^4$ cells/well in 6-well plates followed by isoalantolactone treatment at concentrations ranging from 0–40 μM. After isoalantolactone treatment, a wound was scratched in HEC-1-B cells using sterile pipette tip and left in incubation for 24 h. The anti-migratory efficacy of isoalantolactone was determined by the comparison of wound width in the treated cells to that of the controls.

### Transwell assay

Transwell chambers were used to detect the invasiveness of HEC-1-B cells with/without isoalantolactone treatment. In brief, upper transwell chambers coated with Matrigel were added with a serum free RMPI-1640 medium and lower chambers were fitted with RMPI-1640 medium containing 10% fetal bovine serum. HEC-1-B cells with a density of $1.2\times10^4$ cells/well were loaded to upper chambers, followed by addition of different isoalantolactone concentrations 0–40 μM. Afterwards, transwells were subjected to incubation for 24 h at 37°C, followed by washing off of non-invasive cells with a cotton bud. The invasive HEC-1-B cells were washed in PBS, followed by staining with 0.1% crystal violet. Finally, invasive cells were pictured under a light microscope and compared to controls.

### Western blotting

To determine the expression levels of different proteins western blotting was performed. In brief, the HEC-1-B cells were seeded to above 90% of confluence in 6-well plates at a concentration of $1.2\times10^4$ cells/well. Afterwards, cells received isoalantolactone treatment for 24 h at different concentrations of 0, 10, 20 and 40 μM. Post treatment, HEC-1-B cells were lysed using RIPA lysis buffer followed by protein quantification among lysates using BCA assay. Equivalent amounts of 40 μg of proteins were separated electrophoretically over SDS-PAGE and then loaded over PVDF membranes. These membranes were then blocked with the help of 5% BSA in PBS. Post blocking, membranes were blotted using primary antibodies against MEK, p-MEK, p-ERK,

ERK, Bcl-2, Bax and Actin (1:1000 dilution; Cell Signalling Technology, Beverly, MA, United States) for a time duration of 12 h at 37°C in the dark. Primary antibodies treatment was followed by incubation of membranes with HRP-conjugated anti-mouse secondary antibodies in a dark room for 2 h. Finally, protein bands were detected and visualized using enhanced chemiluminescence reagent.

### Statistical analysis

All experiments were performed in triplicate and results were expressed in terms of mean ± standard deviation (S.D.). Statistical comparison was drawn against controls/within groups with the help of one-way ANOVA followed by multiple comparison analysis using Tukey's test. $P<0.05$ was considered as level for statistical significance.

## RESULTS AND DISCUSSION

### Antiproliferation effects of isoalantolactone against HEC-1-B cells

Around 61880 new endometrial cases and over 12160 deaths due to this malignancy were reported in 2019 (Lorusso *et al.*, 2019). Despite more than 80% of five-year survival in patients, the existing treatment methodology and therapeutics exhibit serious side-effects, which make life of the patients more miserable (Lorusso *et al.*, 2019). Therefore, there is a pressing need to develop efficient chemotherapy for endometrial carcinoma. Consistently, here the anticancer effects of isoalantolactone were evaluated against endometrial HEC-1-B cells. To estimate the antiproliferative effects of isoalantolactone (Fig. 1A) on HEC-1-B and THESC cells, MTT assay was used. After 48 h of isoalantolactone treatment (0-100 μM), significant ($P<0.05$) decrease in the viability of HEC-1-B cells was observed as compared to controls. These antiproliferative effects of isoalantolactone were found to be concentration dependent. An $IC_{50}$ of 10 μM was recorded for isoalantolactone against the HEC-I-B cells (Fig. 1B). In comparison to HEC-1-B cells, isoalantolactone induced very low toxicity to THESCs indicative of the potent antiproliferation effects of isoalantolactone against the HEC-1-B cells (Fig. 1C). It has been reported that cancer cells show aberrant activation/deactivation of different genes and pathways (Stylianou *et al.*, 2006). We believe that isoalantolactone might be targeting some of those pathways, which may explain the sensitivity of HEC-1-B cells to isoalantolactone as compared to the THESC cells.

### Apoptotic cell death induced by Isoalantolactone (μM).

Next mechanistic studies were carried out to unveil the molecular basis of antiproliferative effects of isoalantolactone against HEC-1-B cells. Firstly, the effects of isoalantolactone on cellular apoptosis in HEC-1-B cells was determined. Apoptosis is one of the critical functions of cells which helps in maintaining homeostasis and signifies regular function to discard dysfunctional and excess cells (Carneiro & El-Deiry, 2020). More and more evidence has suggested that apoptosis is involved in maintenance of homeostasis during the period of menstrual cycle through discarding of the senescent cells from uterine endometrium functional layer (Harada *et al.*, ????). Imbalance in the controlled proliferation and normal apoptotic functioning of endometrium cells collectively consequence into neoplasia development (Seebacher *et al.*, 2012). Serious metastatic tumors exhibit strong resistance to apoptosis in comparison to medium metastatic disease. Endometrium cancer cells have been shown to exhibit lesser susceptibility towards apoptosis, besides exhibiting high expressions of survivin gene. Elevated survivin expressions may eradicate apoptosis through caspase-3 and hence contribute to endometrial carcinogenesis (Ai *et al.*, 2006). Hence, we came to know from literature that apoptosis could be a potential target in endometrial cancer treatment. Isoalantolactone has been previously revealed to possess proapoptotic property against wide spectrum of human cancers including glioblastoma (U87), pancreatic cancer (PANC-1) and liver cancer (Hep-G2) (Rasul *et al.*, 2013).

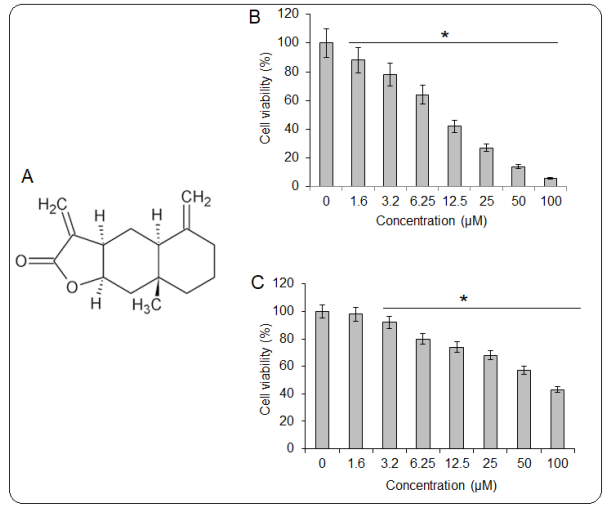


**Figure 1. Isoalantolactone inhibits the proliferation of HEC-1-B endometrial cancer cells.**
(**A**) Chemical structure of isoalantolactone molecule (**B**) MTT assay results represented cellular viability of HEC-1-B cells after exposure to indicated doses of isoalantolactone. Results showed significant inhibition of viability in a concentration-dependent fashion (**C**) MTT assay results showing viability of THESC cells post isoalantolactone exposure. Results of each individual experiment repeated in triplicates were represented as mean ± S.D. *$P<0.05$

Herein, we observed that isoalantolactone induced apoptosis in HEC-1-B cells. As revealed by DAPI staining, indicated characteristic proapoptotic changes in isoalantolactone treated cells include increased nuclear fragments, nuclear pyknosis, chromatin condensation and

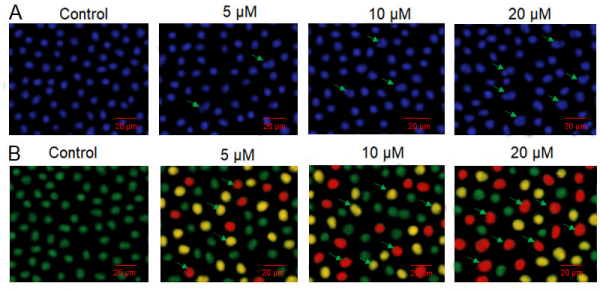


**Figure 2. Isoalantolactone alters nuclear morphology of HEC-1-B endometrial cancer cells**
(A) DAPI staining assay results presenting the apoptotic cell morphology of HEC-1-B cells. Post treatment with isoalantolactone, cells showed damaged nucleus and chromatin condensation. (**B**) AO/EB staining assay results indicating apoptosis induction by isoalantolactone in HEC-1-B cells. Experiments were performed in triplicates.

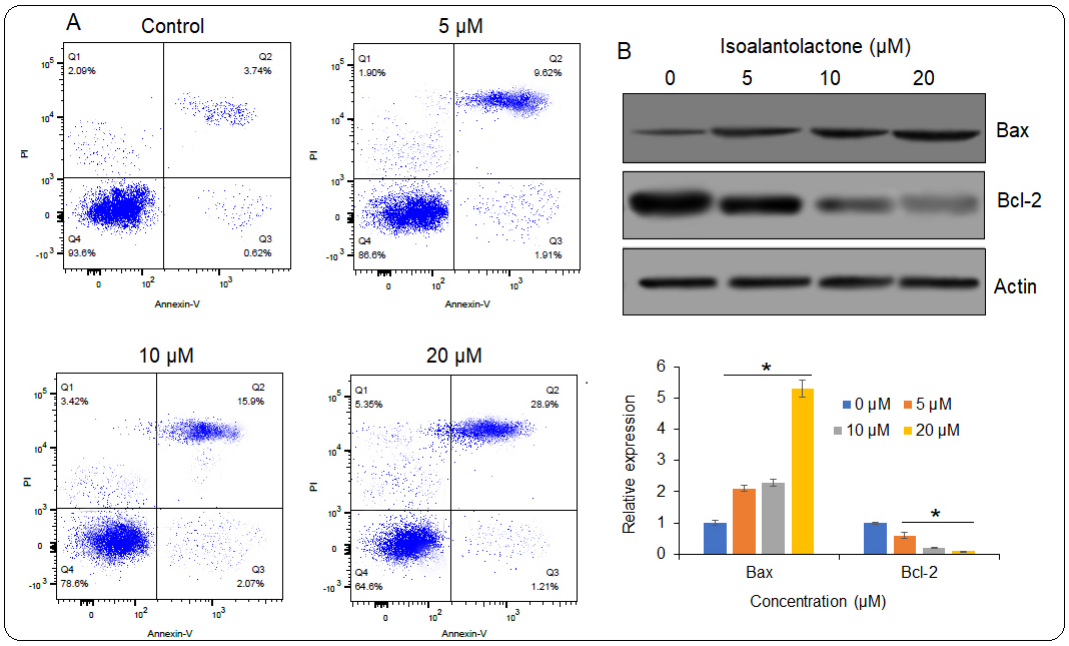


**Figure 3. Isoalantolactone induce apoptosis in HEC-1-B cells.**
(**A**) Annexin V/PI staining assay revealing the quantity of apoptotic cell percentage of isoalantolactone treated HEC-1-B cells. The apoptosis percentage increased dramatically post isoalantolactone exposure (**B**) Western blots presenting the levels of Bax and Bcl-2 proteins in HEC-1-B cells indicating proapoptotic effects of isoalantolactone. Experiments were performed in triplicates and represented as mean ± S.D. **P*<0.05

irregular edges around the nucleus. Meanwhile, the control cells revealed regular, clear edged and round nucleus (Fig. 2A). Therefore, DAPI staining presented proapoptotic effects of isoalantolactone in HEC-1-B cells. Further, AO/EB staining assay showed that isoalantolactone induced proapoptotic morphological changes in the nucleus of the treated HEC-1-B cells. Major percentage of cells in the treated groups revealed fragmented and condensed chromatin with bright orange fluorescence (Fig. 2B). Control cells showed bright green fluorescence without any significant change in nuclear morphology. We further quantified the apoptosis in HEC-1-B cells with/without isoalantolactone treatment (0–40 μM). Results showed that the treatment group showed significant ($P<0.05$) rise in the number of apoptotic cells than that of control cells (Fig. 3A). Annexin V/PI assay showed that the percentage of the apoptotic HEC-1B cells increased from 3.7% in untreated cells to 28.9% at 20 μM of isoalantolactone. To further validate apoptotic effects of isoalantolactone in HEC-1-B cells, we performed western blotting assay to determine the expression of Bax and Bcl-2. The results indicated that the isoalantolactone treated groups have elevated expressions of Bax (proapoptotic protein) and reduced expressions of Bcl-2 (antiapoptotic protein) in comparison to control (Fig. 3b). The apoptosis inducing effects of isoalantolactone were found to follow a concentration-dependent fashion.

## ROS production enhanced by isoalantolactone.

Oxidative stress induced *via* ROS production has been found to interfere with almost all stages of oncogenic processes such as initiation, promotion and progression of carcinogenesis (Waris & Ahsan *et al.*, 2006). ROS alters the expression levels of different transcription factors during developmental stage of cancer like proliferation, mutations, inhibition, senescence and differentiation (Klotz & Steinbrenner, 2017). As ROS is involved in carcinogenesis, these can also bring about cell death via stimulation of different cellular mechanisms in cancer cells, including apoptosis. ROS have been also recognized with active participation in progression of endometriosis and endometrial cancer (Kim *et al.*, 2018). Therefore, ROS serves as a potential target for therapeutics against endometrial cancer.

Isoalantolactone was previously shown to possess extraordinary property of ROS production in cancer cells thereby mediating oxidative-stress linked apoptosis (Khan *et al.*, 2012). Herein, our findings demonstrated that isoalantolactone caused tremendous elevations of ROS in HEC-1-B cells, as indicated by DCFH-DA staining (Fig. 4). With the increase in isoalantolactone concentrations, significant increase in DCF-fluorescence is observed in HEC-1-B cells. The ROS levels increased to 210% of the control at 20 μM isoalantolactone. Taken together, the results suggest isoalantolactone could initiate HEC-1-B cell death *via* oxidative-stress mediated apoptosis.

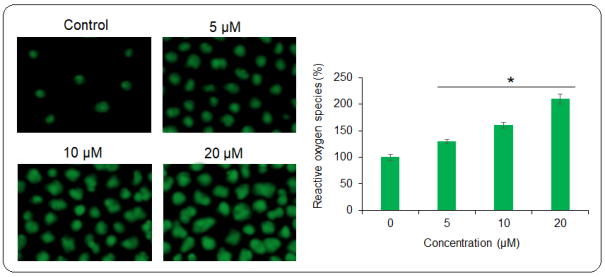


**Figure 4. The intracellular ROS were examined by DCFH-DA staining assay.**
Results showed strong burst of ROS product in treated cells compared to control group. Experiments were executed in triplicates presented as mean ± S.D. **P*<0.05

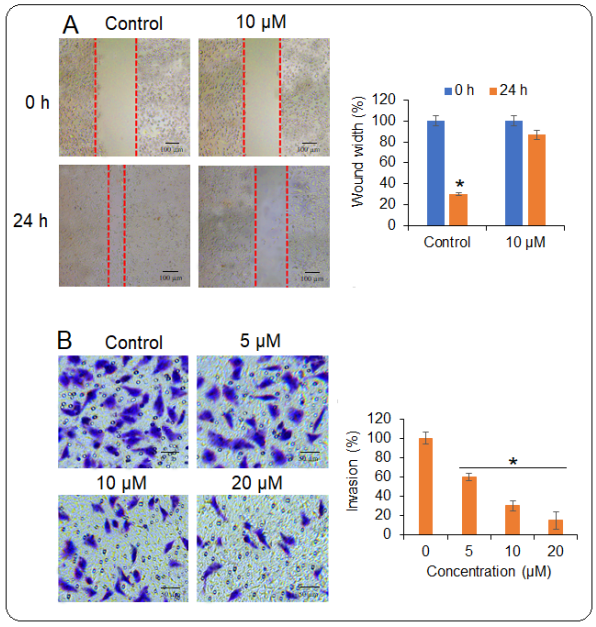


**Figure 5. Isoalantolactone inhibits the migration and invasion of HEC-1-B cells**
(**A**) The wound healing assay results showing the wound closure before and after isoalantolactone treatment of HEC-1-B cells. (**B**) Transwell migration assay indicating the invasiveness of HEC-1-B cells pre and post isoalantolactone exposure. Experiments were executed in triplicates presented as mean ± S.D. *$P$<0.05

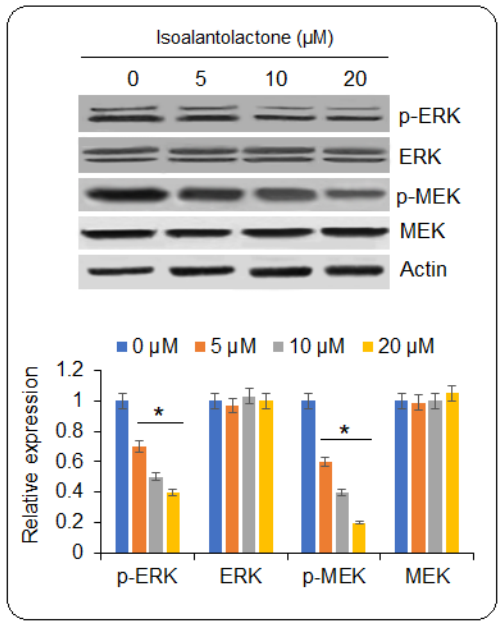


**Figure 6. Isoalantolactone blocks MEK/ERK pathway.**
Western blots displaying the expressions of the MEK/ERK signalling related proteins in isoalantolactone treatment and control groups. Experiments were executed in triplicates.

### Inhibition of migration and invasion by isoalantolactone

Next, isoalantolactone has been identified with significant potential to target migration and invasion of cancer cells, including that of breast cancer MDA-MB-231 cells (Wang *et al.*, 2016). Migration and invasion are the two lethal features of cancer cells enabling them to transform to metastatic cancer. The late-stage endometrial cancer also shows the characteristic of metastasis. Therefore, inhibition of these two mechanisms of cancer cells could prove a leading strategy in overcoming metastatic endometrial cancer. Herein, we found that isoalantolactone caused a significant arrest of migration (Fig. 5A) as well as invasion (Fig. 5B) of HEC-1-B cells.

### Suppression effects of isoalantolactone on Ras/Raf/MEK signalling pathway

The MEK/ERK signalling is an important pathway in maintenance of proper functioning of cancer cells and also regulates their proliferation and differentiation (Degirmenci *et al.*, 2020). MEK/ERK signalling cascade gets stimulated by different upstream signalling sources like mitogen, interleukin, cytokines, growth factors and genetic mutations. These sources in endometrial cancer could interact with membrane receptors like RTK's (Chi *et al.*, 2019). MEK/ERK signalling has been found to be over-activated in endometrial cancer due to the fact that receptors show overexpression as well. The K-RAS gene has been often reported in 20% of endometrial cancers, it encodes a small GTPase superfamily protein (Dobrzycka *et al.*, 2009). Therefore, due to over-activity of MEK/ERK signalling in endometrial cancer cells, drugs that target this signalling could be beneficial in overcoming this malignancy. The effects of isoalantolactone against MEK/ERK signalling in endometrial HEC-1-B cells were evaluated for the first time in this study. Results showed that isoalantolactone inhibited both phosphorylation of MEK and ERK in a concentration-dependent fashion (Fig. 6). Therefore, these results indicate that Ras/Raf/MEK signalling could be involved in proapoptotic cell death of HEC-1-B cells by isoalantolactone.

## CONCLUSION

Altogether, the results of this research revealed potent anti-cancer property of isoalantolactone molecule against HEC-1-B cells. The anticancer effects of isoalantolactone were found to arbitrate via ROS mediated apoptosis and inhibition of MEK/ERK signalling. Furthermore, isoalantolactone exhibits anti-migratory and anti-invasive effects against the HEC-1-B cells. Therefore, isoalantolactone may prove to be an effective therapeutic agent against endometrial cancer. However, more research endeavors, especially *in vivo* studies are required to establish isoalantolactone as lead molecule for the development of chemotherapy for endometrial cancer.

### Conflict of interest

Authors declare that there are no conflicts of interest.

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