



# Withaferin A inhibits proliferation of human endometrial cancer cells via transforming growth factor- $\beta$ (TGF- $\beta$ ) signalling

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## Abstract

The present study was designed to evaluate the anticancer effects of withaferin A against the human endometrial cancer via modulation of transforming growth factor- $\beta$  (TGF- $\beta$ ) signalling. The results of the present study revealed that withaferin A exerts a dose and time-dependent antiproliferative effects against the human KLE endometrial cancer cells with comparatively lower toxicity against the THESCs normal cells. The IC<sub>50</sub> of withaferin A against the KLE endometrial cancer cells was found to 10  $\mu$ M. The results showed that withaferin A induced apoptosis and G<sub>2</sub>/M cell cycle arrest of the KLE cells which was associated with alteration of the apoptosis and cell cycle related proteins. In addition, the transwell assays showed that the migration and invasion of the KLE cells were inhibited by 53 and 40%, respectively. Finally, the effects of withaferin A were also examined on the TGF- $\beta$  signalling pathway. The results showed that withaferin A blocked TGF- $\beta$ -dependent Smad2 phosphorylation and expression of other TGF- $\beta$ -related proteins in KLE cells. Summing up, the results suggest that withaferin A inhibits the proliferation of the human endometrial carcinoma via TGF- $\beta$  signalling.

**Keywords** Endometrial cancer · Withanolides · Withaferin A · Apoptosis · TGF- $\beta$  signalling

## Introduction

Endometrial carcinoma comprises of an assembly of distinctive histological subtypes which collectively constitute the most frequent gynaecological disorder across the globe (Green et al. 2020). Alone in United States, about 12,590 deaths and 65,620 new cases of endometrial carcinoma were reported in 2020 (Siegel et al. 2020). Prognosis of endometrial carcinoma is highly based on the clinical stage and histological grade (Torre et al. 2015). Even though, patients with early-stage disease (67%) show 5-year overall survival of above 81%, patients with later stages IVA and IVB endometrial carcinoma show an extremely poor prognosis of 17 and 15%, respectively (Constantine et al. 2019). Mortality associated with endometrial carcinoma is increasing at an alarming pace. From 2005 to 2014, an approximately 1.4% increase in death rate associated with this malignancy was recorded (Siegel et al. 2020; Torre

et al. 2015). The key risk factors contributing to the carcinogenesis of endometrial carcinoma includes tamoxifen exposure, obesity, nulliparity, late menopause and early menarche (Raglan et al. 2019; Constantine et al. 2019; Morice et al. 2016). Obesity is one of the leading and modifiable risk factors in endometrial carcinoma accounted for about 50% of cases in United States and Europe. Obesity in endometrial carcinoma patients increases the death risk by almost 6.25 times (Calle et al. 2003). The treatment methodologies for patients with initial-stage endometrial carcinoma include radiation therapy and/or chemotherapy. Patients suffering from recurrent and advanced stage endometrial carcinoma are treated with targeted therapy, cytotoxic therapy or hormonal therapy (Morice et al. 2016). Because of extremely poor prognosis for recurrent and advanced stage disease, the hunt for novel chemopreventives is the need of the hour. Owing to immense structural diversity of natural products, they constitute a remarkable source of drugs (Ji et al. 2009; Majolo et al. 2019). Several natural products have been reported to exhibit strong anticancer activities and some of them have even been already reached clinical trials (Newman 2018). Withanolides belong to naturally occurring steroid lactones class of compounds mostly found in *Withania* genus (Dhami

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et al. 2017). Withanolides exhibit remarkable biological and medicinal properties, including anti-inflammatory, antistress, hepatoprotective, antioxidant, immunomodulatory, adaptogenic, antibacterial, insect antifeedant, antiarthritic and anticancer (Singh et al. 2010). The molecule withaferin A, belongs to medicinally active members of withanolides, has been reported to possess strong biological applications, including antiangiogenesis, immunomodulatory, antioxidant, anti-inflammatory, antistress and anticancer activities (vel Szic et al. 2014). Moreover, withaferin A is turning out to be a leading anticancer agent against pancreatic, prostate, uterine and breast cancers (Lee and Choi 2016). The anticancer potential of withaferin A and need for therapeutic agents for endometrial carcinoma prompted us to evaluate the anticancer effects of withaferin A against KLE endometrial carcinoma cells. In addition, attempts were made to unveil the underlying mechanisms associated with withaferin A triggered anticancer effects.

## Materials and methods

### Cell lines, culture and conditions

The cancerous KLE endometrial cells and normal THESCs endometrial cells were procured from American Type Culture Collection (ATCC, Manassas, VA, United States). Withaferin A (> 98% purity by HPLC) was purchased from Sigma Aldrich (St. Louis, MO, United States). All the cell lines were cultured in RMPI-1640 medium containing high glucose (4.5 g/L), 2 mM L-glutamine, Penicillin (50 IU/mL), Streptomycin (50 mg/mL) and 10% fetal bovine serum. All the reagents and medium were purchased from Sigma Aldrich (St. Louis, MO, United States) or else otherwise mentioned. Both the cell lines were maintained under humid conditions at 37 °C in a 5% CO<sub>2</sub> and 95% air.

### Cell viability assay

The viability of KLE and THESCs cells treated with withaferin A was examined by 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazoliumbromide (MTT) assay. Concisely, each cell line with a density of  $2 \times 10^5$  cells/well in 96-well plate were exposed to different withaferin A concentrations viz 0, 2.5, 5, 10, 20, 40, 80 and 180  $\mu$ M for 48 h at 37 °C. Post-treatment, 0.5% MTT reagent (Sigma) was supplied to each well and incubation was performed at 37 °C for additional 4 h. Thereafter, dimethyl sulphoxide (DMSO) was added to each well followed by processing

of the cell samples for absorbance measurements to determine cell proliferation at 570 nm.

### Cell proliferation assay

The proliferation of the KLE cells was determined by EdU (5-ethynyl-2'-deoxyuridine) assay. Briefly, the KLE cells ( $2 \times 10^5$  cells/well) were treated with 10  $\mu$ M concentration of withaferin A within 96-well plates maintaining EdU medium for 48 h. EdU assay was performed using EdU assay kit (Ribobio). DAPI staining was used to observe cell nuclei and EdU-positive cells were examined under a fluorescence microscope.

### Annexin-V/PI assay

Cancer cell apoptosis was studied through Annexin-V/PI staining assay after withaferin A treatment. In brief, KLE cells were placed in 12-well plates ( $10^5$  cells/well) and treated with 0 and 10  $\mu$ M concentrations of withaferin A for 48 h. Cells were then harvested through centrifugation followed by PBS washing. Post-washing, cells were fixed in ethanol (70%) and then staining was performed using Annexin V-FITC and propidium iodide (PI). Staining was performed by following manufacturers protocol of eBioscience™ Annexin V Apoptosis Detection Kit FITC (Thermo Fisher Scientific). Finally, apoptosis analysis was performed via flow cytometry.

### Immunofluorescence analysis

Withaferin A treated (0 and 10  $\mu$ M) KLE cells were fixed for 15 min in paraformaldehyde (4%) followed by incubation of 30 min with Triton X-100 (Beyotime, China). Subsequently, cells were subjected to incubation overnight at 4 °C with anti-cytochrome C antibody (1:100 dilutions, proteintech, China). Afterwards, cells were washed using PBS followed by incubation with Cy3 goat anti-rabbit IgG (1:200 dilutions, Beyotime, China). Subsequently, the cells were stained with DAPI (Biosharp, China) and finally examined under a fluorescent microscope.

### Flow cytometry

Flow cytometry was used to determine the effects of withaferin A on distribution of the KLE cells in different phases of cell cycle. Concisely, KLE cells were placed in 12-well plates ( $10^5$  cells/well) and treated with 0 and 10  $\mu$ M concentrations of withaferin A for 48 h. Thereafter, the cells were fixed and subsequently incubated with RNase (100  $\mu$ L of concentration 100  $\mu$ g/mL) and 400  $\mu$ L of PI dye at 4 °C. Finally, filtration was carried out with 300 mesh (70  $\mu$ m)

cell strainer and ultimately subjected to flow cytometry for cell cycle analysis.

### Transwell assay

Cell migration and invasion ability of KLE cells post-withaferin A exposure was measured by using transwell assay. KLE cells were exposed to 0 and 10  $\mu\text{M}$  concentrations of withaferin A for 24 h followed by loading of  $2 \times 10^3$  cells to upper transwell chamber maintaining serum-free RMPI-1640 medium. RMPI-1640 medium maintaining 10% FBS was supplied to lower chamber. Afterwards, chambers were incubated at 37 °C for 24 h followed by clearing of cells on upper surface of the membrane with a cotton swab. The migrated KLE cells underside of the membrane were subjected to staining for 10 min using crystal violet (0.1%). The cells, which migrated were finally counted and pictured at 200 $\times$  magnification field of a light microscope. Consistent procedure was followed for invasion evaluation except transwell chambers were coated with Matrigel.

### Western blotting

Total proteins from withaferin A treated (0 and 10  $\mu\text{M}$ ) KLE cells was isolated using RIPA lysis buffer. Proteins were thereafter separated electrophoretically on SDS-PAGE. Afterwards, separated proteins were blotted to PVDF membranes followed by specific primary antibodies treatment overnight at 4 °C. Following primary antibody treatment, the membranes were exposed to a secondary antibody. Finally, visualization of protein bands was performed with the help of enhanced chemiluminescence substrate on X-ray films.

### Statistical analysis

Experiments were performed in triplicate and expressed as mean  $\pm$  standard deviation (SD). Statistical analysis was carried out by performing different statistical tests on SPSS 15.0 software. The measure of statistically significant difference was taken at  $p < 0.05$ .

## Results

### Withaferin A inhibited KLE cell proliferation

To assess the viability and proliferation of normal THESCs and cancerous KLE cells (Fig. 1A), we used MTT assay and EdU assay. The results from MTT assay showed that withaferin A exhibits significant ( $p < 0.05$ ) anti-proliferative effects against KLE cells. The viability of THESCs cells post-48 h prolonged withaferin A treatment (0–160  $\mu\text{M}$ ) was observed to be decreased from 100% to almost 25% with an  $\text{IC}_{50}$  value

of 76  $\mu\text{M}$  (Fig. 1B). In case of endometrial cancer KLE cells, withaferin A showed caused significant inhibition of KLE cell proliferation with an  $\text{IC}_{50}$  value of 10  $\mu\text{M}$  (Fig. 1C). Furthermore, withaferin A exerted time-dependent anticancer activity against the KLE cells (Fig. 1D). The results of EdU assay revealed that withaferin A caused remarkable inhibition of KLE cell proliferation (Fig. 1E). Collectively, withaferin A induced potent antiproliferative effects against the human endometrial cancer cells.

### Withaferin A promoted mitochondria-mediated-apoptosis in KLE cells

To detect the apoptosis in KLE cells after withaferin A treatment, annexin-V/PI staining was performed. The results showed that post-withaferin A exposure the percentage of early-stage and late-stage apoptotic cells increased significantly. In comparison to 12.25% of apoptotic cell percentage in control, withaferin A (10  $\mu\text{L}$ ) treated cells showed 40.1% of apoptotic cells (Fig. 2A). The results of western blotting indicated that the expression of Bax increased and that of Bcl-2 decreased. In addition, withaferin A treatment increased the cleavage of caspase-3 and caspase-9 (Fig. 2B). Caspase-3 and -9 are the key apoptosis allied proteins that modulate discharge of cytochrome-C into cytoplasm. Therefore, we further performed immunofluorescence analysis to monitor discharge of cytochrome-C. The results showed increased expression of cytochrome-C in withaferin A treated KLE cells in comparison to that of control untreated cells (Fig. 2C). These results indicated that withaferin A promoted apoptosis of KLE cells.

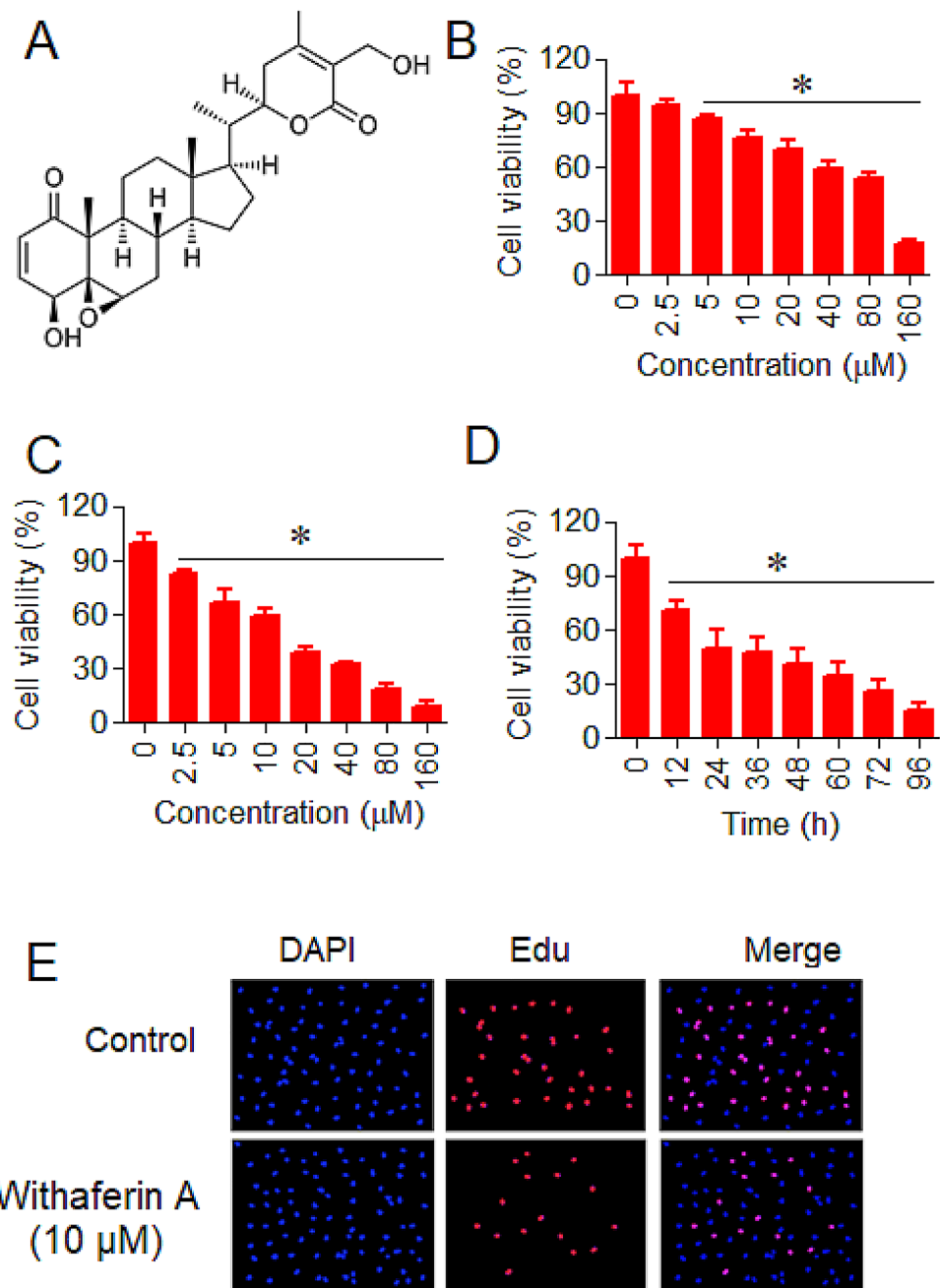
### Withaferin A targeted cell cycle in KLE cells

To monitor the distribution of KLE cells in different cell cycle phases treatment with withaferin A at 0 and 10, flow cytometry was performed. The results that withaferin A triggered the accumulation of the KLE cells in the  $G_2/M$ -phase of the cell cycle. The  $G_2/M$ -phase cell percentage in control group was 4.68% which enhanced to 25.97% in withaferin A treated group (Fig. 3A). These results indicate that withaferin A induces  $G_2/M$  phase arrest of the KLE cells. Further, we examined the expression of cell cycle allied proteins in KLE cells post-withaferin A treatment. The results showed decrease in the expression of cdc2, cdc25c and cyclin-B1 in KLE cells.

### Withaferin A repressed migration and invasion of KLE cells

The transwell assay was performed to detect the effects of withaferin A on migration and invasion of KLE cells. The results indicated withaferin A strongly repressed the

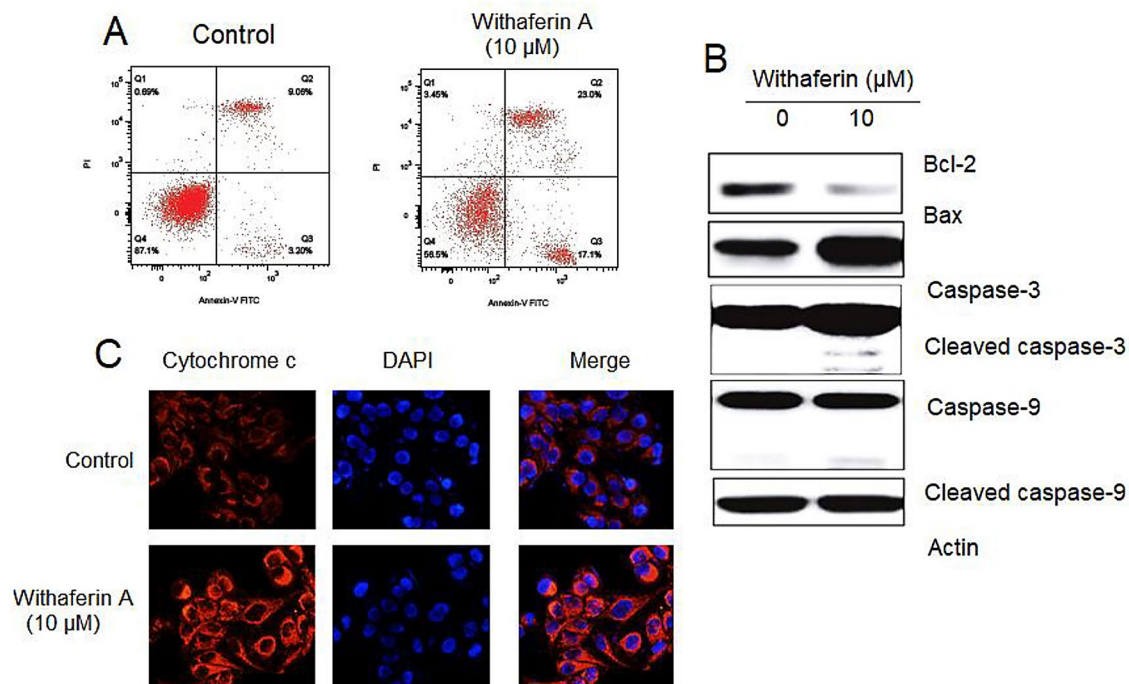
**Fig. 1** Withaferin A inhibits the viability and proliferation of endometrial carcinoma cells. **A** Chemical structure of withaferin A molecule. **B** Proliferation of THESCs cells post-withaferin A treatment at indicated concentrations. **C** Proliferation of KLE cells post-withaferin A treatment at indicated concentrations. **D** Proliferation of the KLE cells at  $IC_{50}$  concentration and at different time periods as indicated. **E** Proliferation of KLE cells post-withaferin A treatment as depicted by EdU assay. Three replicates were of experiment were performed and final data were presented as mean  $\pm$  standard deviation. (\* $p < 0.05$ )



migration and invasion potency of KLE cells. Withaferin A repressed the migration percentage from 100% (control) to 53% (treated group) (Fig. 4A). Similarly, withaferin A treatment (0 and 10  $\mu$ M) to KLE cells resulted in decrease of invasive cell percentage from 100% to almost 40% (Fig. 4B). Moreover, western blotting showed decrease in the expression of MMP-2 and MMP-9 in KLE cells post-withaferin A treatment (Fig. 4C).

#### Withaferin A inhibited TGF- $\beta$ signalling in KLE cells

To evaluate the effects of withaferin A on TGF- $\beta$  signalling in KLE cells, western blotting assay was performed. The results showed that the expression of TGF- $\beta$ 1, TGF- $\beta$ 2, p-Smad2 and p-Smad3 were decreased considerably. Nonetheless, Smad2 and Smad3 remained all-over constant (Fig. 5). This indicated that the antiproliferative and



**Fig. 2** Withaferin A induces apoptosis in KLE cells. **A** Apoptosis analysis of withaferin A treated KLE cells through Annexin V/PI staining. **B** Western blots representing expressions of apoptosis associated proteins. Bax, Bcl-2, cleaved caspase-3, caspase-3 and cleaved

caspase-9 and caspase-9. **C** Immunofluorescence analysis indicating expressions of cytochrome-C in withaferin A treated KLE cells. Experiments were repeated thrice

proapoptotic effects of withaferin A are mediated by down-regulation of TGF- $\beta$  signalling in KLE cells.

## Discussion

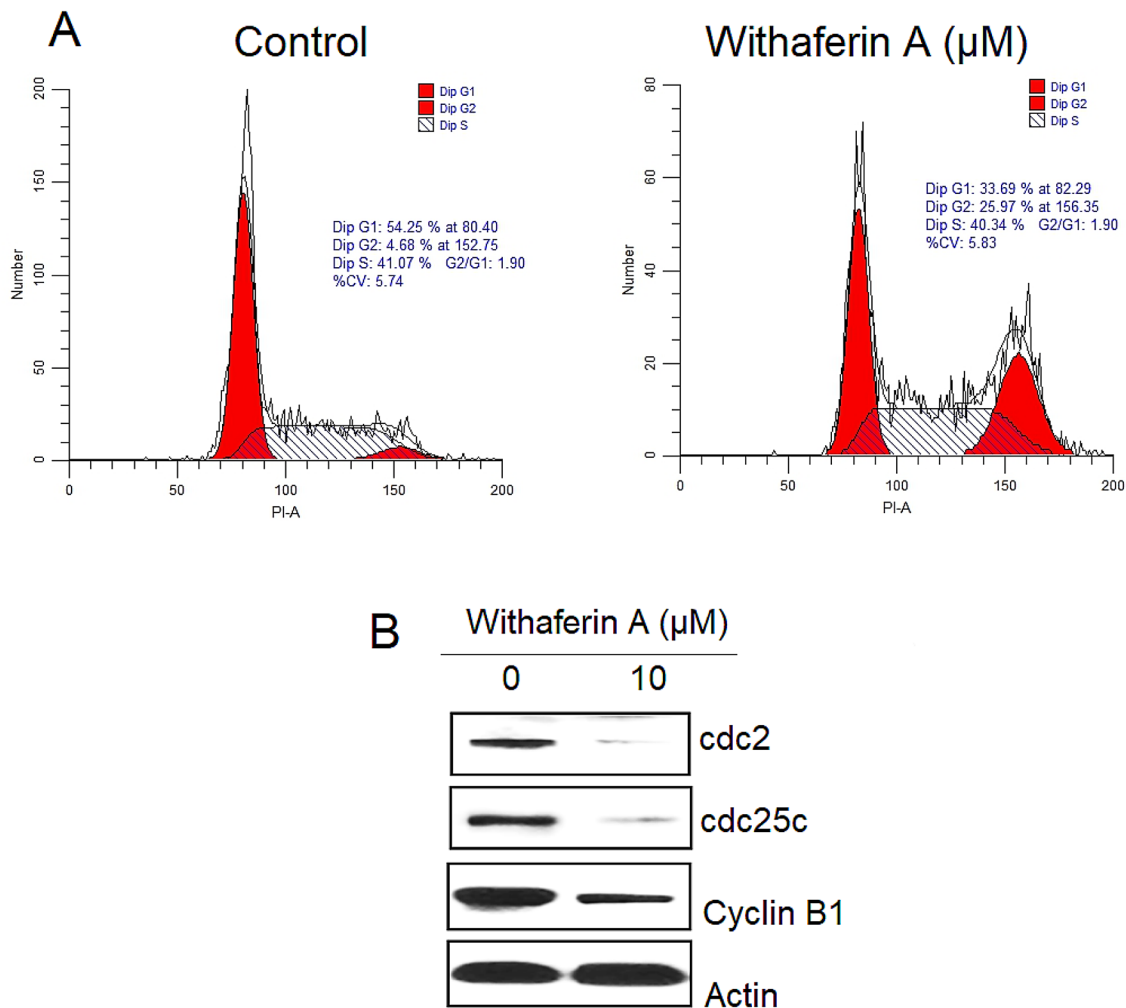
Unfortunately, the endometrial carcinoma incidences and mortality pose a huge threat to female health, globally. Although the recent advancements have led to the development of efficient treatment methodologies and biomarkers for early detection of disease, the overall survival rates for later-stage endometrial carcinoma still remain far from descent. Natural products are ranked as essential modulatory molecules in eukaryotic organisms because they mimic several biological components found in these organisms (Al-Awadhi and Luesch 2020). They have been reported to exert remarkable influence on several physiological processes, developmental processes and health in human beings. Natural products target key survival regulatory proteins in cancer cells to inhibit their proliferation via different molecular mechanisms, such as apoptosis and autophagy (Wang and Feng 2015).

Withaferin A is a medicinally high rated natural product shown with strong antiproliferative effects against several human cancer cells, including colon, gastric, prostate and pancreatic cancer (Lee and Choi 2016). Herein, the

antiproliferative effects of withaferin A were evaluated against KLE cells. Withaferin A demonstrated strong proliferation inhibitory effects against KLE cells ( $IC_{50}$  value of 10  $\mu$ M) in a concentration- and time-reliant mode. Cytotoxicity of withaferin A was comparatively lower against normal THESCs endometrial cells ( $IC_{50}$  value of 76  $\mu$ M) when compared to cancerous KLE cells.

On establishment of antiproliferative effects of withaferin A against KLE cells, further studies were carried out to determine the underlying molecular mechanism. Programmed cell death (apoptosis) is a highly organized process in which the intracellular content is collected in membrane packets for destruction by immune cells (Elmore 2007). Apoptosis maintains normal balance in multicellular organisms by eliminating malfunctioning, damaged, virus-infected or cancerous cells during development. Herein, withaferin A demonstrated pro-apoptotic activity in KLE cells which was supported by upregulation of pro-apoptotic proteins Bax, downregulation of Bcl-2 and triggered cleavage of caspase-3 and 9. Moreover, immunofluorescence analysis revealed discharge of cytochrome-c into cytoplasm post-withaferin A exposure of KLE cells. This indicated that withaferin A induces intrinsic apoptosis in KLE cells. Our results were in consistency with the previous studies where withaferin A was shown to regulate apoptosis in human melanoma and leukemia cancer cells by targeting the key modulatory





**Fig. 3** Withaferin A induces  $G_2/M$  cell cycle arrest. **A** Flow cytometric analysis for illustration of different cell cycle phases. The figure represented augmented number  $G_2/M$ -phase cells in treated group

as compared to controls. **B** Western blots showing the expression of cell cycle associated proteins in withaferin A treated KLE cells. The experiments were performed in triplicate

processes and proteins including activation of caspase-3 and -9, DNA cleavage, mitochondrial membrane potential loss, cell cycle arrest, upregulation of Bax and downregulation of Bcl-2 (Mayola et al. 2011; Mandal et al. 2008).

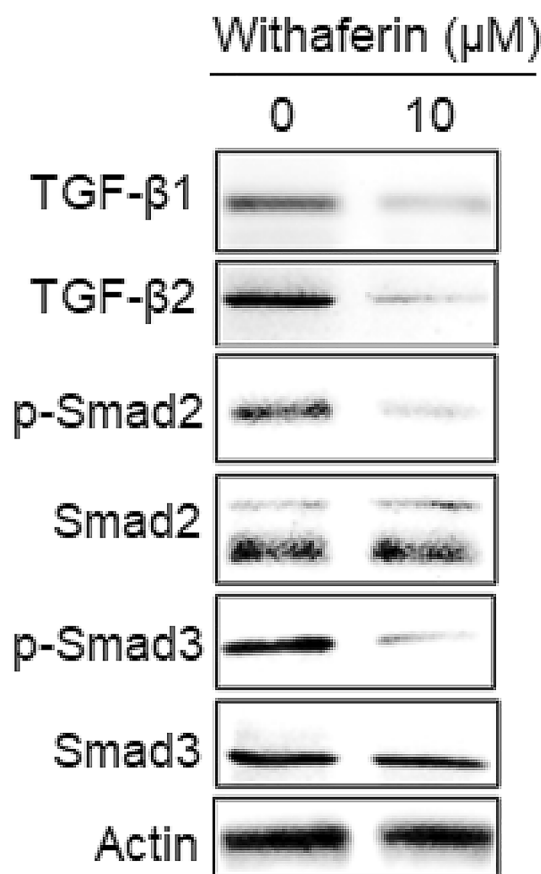
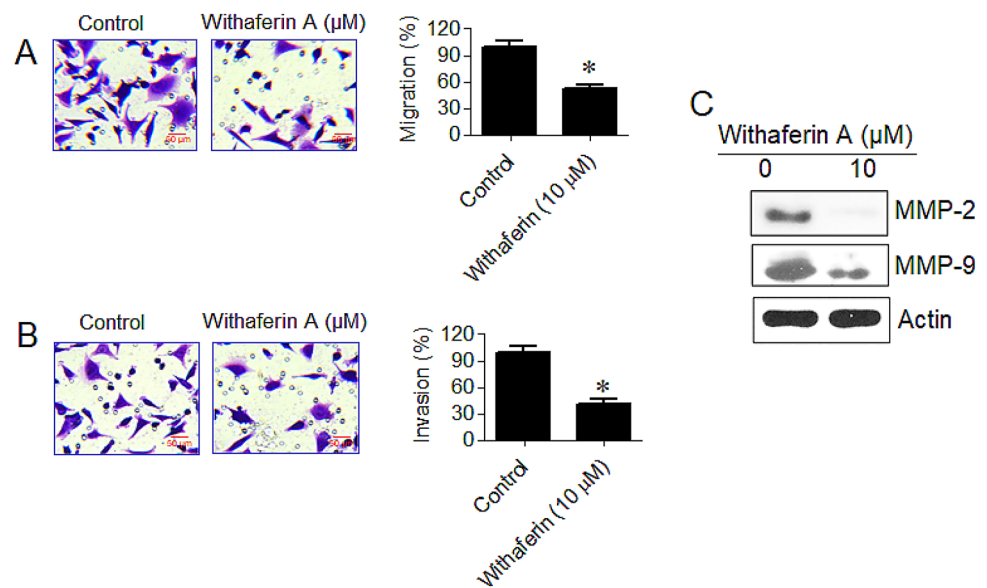
Uncontrolled and rapid cell division is one of the key features of cancer cells (Marte 2004). Cell cycle proceeds through several check points during its course. These check points serve as main targets for chemopreventives to suppress rapid cell division of cancer cells. Herein, withaferin A showed significant inhibition of cell division at  $G_2/M$ -phase of KLE cell cycle. Further, western blotting showed that withaferin A reduced the expression levels of cell cycle allied proteins including cyclin-B1, cdc25c and cdc2. Withaferin A has been already reported to exert cell cycle inhibitory effects against different cancer cells. Withaferin A has been shown to inhibit progression of cell cycle in breast and prostate cancer cells at  $G_2/M$ -phase via downregulation

of cdc25b, cdc25c and cdc2 (Stan et al. 2008; Roy et al. 2013).

Cell migration and invasion play critical role in cancer metastasis. Withaferin A has been previously reported of anti-migration and anti-invasion effects against oral carcinoma cells (Yu et al. 2020). Herein, withaferin A showed significant inhibition of migration as well as invasion of KLE cells. Moreover, post-withaferin A exposure the expressions of MMP-2 and MMP-9 reduced remarkably in KLE cells indicating inhibition of migration and invasion.

TGF- $\beta$  signalling is an important pathway that plays a crucial role in fibrosis, cell growth, differentiation and apoptosis (Morris et al. 2015). Cancer cells mostly show elevated TGF- $\beta$  signalling. TGF- $\beta$  signalling is modulated by non-Smad and Smad pathways regulated by TGF- $\beta$  ligands, type-1 and type-2 receptors and non-Smad or Smad proteins (Liu et al. 2018). Mammals have three

**Fig. 4** Withaferin A inhibits migration and invasion of KLE cells. **A** Transwell migration assay. Results showing repressed migration in withaferin A treated KLE cells in comparison to controls. **B** Transwell invasion assay. Results showing repressed invasion in withaferin A treated KLE cells in comparison to controls. **C** Western blots representing expressions of cell cycle associated proteins. Results revealed decreased expressions of MMP-2 and MMP-9 in KLE cells post-withaferin A treatment. Three replicates were given to all experimental procedure and final data were presented as mean  $\pm$  standard deviation. (\* $p < 0.05$ )



**Fig. 5** Withaferin A blocks the TGF- $\beta$ 1 pathway. Western blots displaying the activity of TGF- $\beta$  signalling in withaferin A treated KLE cells. The activity of TGF- $\beta$ 1, TGF- $\beta$ 2, p-Smad2 and p-Smad3 were downregulated and Smad2 and Smad3 remained almost constant. The experiments were performed in triplicate

TGF- $\beta$  types including TGF- $\beta$ 1, TGF- $\beta$ 2 and TGF- $\beta$ 3. Herein, withaferin A induced inhibition of TGF- $\beta$  signalling through Smad pathway. The expressions of TGF- $\beta$ 1, TGF- $\beta$ 2, p-Smad2 and p-Smad3 were downregulated and Smad2 and Smad3 remained almost constant in KLE cells post-withaferin A exposure. Our results were similar to that of Bale et al., wherein withaferin A induced inhibition of TGF- $\beta$ /Smad signalling in scleroderma (Bale et al. 2018).

In conclusion, we demonstrated that withaferin A induced time and dose-dependent anticancer effects against human endometrial cancer cells. We further established the mechanism by which anticancer effects of withaferin A are regulated. The results revealed Withaferin A stimulated apoptosis, blocked cell cycle, inhibited migration and invasion and targeted TGF- $\beta$  signalling in KLE cells.

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**Author contribution** Conceptualization: KX and JQ; methodology: KX, HS and YD; formal analysis and investigation: KX, HS and YD; writing—original draft preparation: YX, HS and YD; writing—review and editing critically for important intellectual content: JQ; supervision: JQ.

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## Declarations

**Conflict of interest** All the authors declare that he has no conflict of interest.

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