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ORIGINAL ARTICLE

Taraxastane inhibits the proliferation, migration and invasion of human cervical cancer by inducing ROS- mediated necrosis like cell death, cell cycle arrest and modulation of JNK/MAPK signaling pathway

Junbo Hu[1], Yanli Zhang[1], Peng Guo[1], Na Tang[1], Yanju Lu[1], Zhichun Jin[2]

[1]Department of Pathology, Maternal and Child Health Hospital of Hubei Province, Wuhan City, Hubei Province, China, 430070.
[2]Department of Traditional Chinese Medicine, Maternal and Child Health Hospital of Hubei Province, Wuhan City, Hubei Hospital, 430070, China.

Summary

Purpose: *A good number of anticancer studies have focused on the evaluation of plant derived natural products against different types of human cancers. Triterpenes, belonging to terpenoid class of plant secondary metabolites, have been reported to function as potent anticancer agents. The current study was designed to investigate the anticancer potential of Taraxastane against human cervical cancer cells.*

Methods: *MTT assay and DAPI staining were used for determining the cell viability. DCFH-DA and $DiOC_6$ based estimations were employed for determination of reactive oxygen species (ROS) and mitochondrial membrane potential (MMP), respectively. Flow cytometry technique was used for analysis of cell cycle and necrosis. Analysis of cell migration and invasion was performed by wound heal and transwell assays, repectively. Protein expression was analyzed by Western blotting.*

Results: *MTT assay showed that Taraxastane inhibited the proliferation of DoTc2 cervical cancer cells in a concentration-dependent manner with an IC_{50} of 20μM, whereas it had lesser effect on the proliferation of normal human cervical cells. The molecule was seen to increase the ROS concentration along with decreasing MMP. Flow cytometry showed that Taraxastane didn't induce cell apoptosis in DoTc2 cells and anticancer effects were mainly in the form of DoTc2 cell necrosis. This was also evident from the western blot analysis of Bax and BCl-2 proteins whose concentration remained unchanged under Taraxastane treatment. Taraxastane treatment led to cell cycle arrest at G2/M checkpoint without any effect on Cyclin D protein expression. Western blotting of JNK and p-38 proteins showed that Taraxastane blocks the JNK/MAPK signaling pathway by preventing the phosphorylation of the former in a dose-dependent manner. Finally, the wound healing and transwell assays showed that Taraxastane inhibited the migration and invasion of cervical cancer cells, which indicates the role of Taraxastane in the prevention of cancer metastasis.*

Conclusion: *To conclude, Taraxastane has remarkable antiproliferative effect on human cervical cancer cells and thus may prove as a vital lead molecule for discovery of anticancer drugs.*

Key words: *triterpenes, Taraxastane, cervical cancer, cell cycle arrest, necrosis, invasion, metastasis*

Introduction

Cervical cancer is the fourth most commonly occurring and also the fourth most death-causing cancer among women worldwide. According to an estimate, about 528,000 cases of cervical cancer occurred in 2012, leading to about 266,000 deaths [1]. This accounts to about 8% of the total cancer cases and mortality from cancer [2]. Developing countries have nearly 70% occurrence of cervical cancer with

Corresponding author: Zhichun Jin, Department of Pathology, Traditional Chinese Medicine, Maternal and Child Health Hospital of Hubei Province, No.745 Wuluo Rd , Wuhan City, Hubei Province, China, 430070.
Tel/Fax: +86 027 87161012, Email: JanelleStonetnp@yahoo.com
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about 90% deaths [1,3]. Although the pervasive usage of cancer screening programs have noticeably reduced the rates of cervical cancer in developed countries [4], it is still one the leading cause of cancer-related death in lower economy countries. Therefore, efficient treatment strategies are the need of hour to restrain the incidence of this disease. Plants, being important natural reservoirs of a vast array of biologically active chemicals, have been employed for the usage of a number of secondary metabolites against different types of cancers [5,6]. Triterpenes, the members of isoprenoids derived from a C30 precursor squalene, exhibit wide structural diversity and biological activity [7]. They have been found to act as potential anticancer agents, for instance, Betulinic acid, a pentacyclic triterpene in the bark of white birch trees, is a selective inhibitor of melanoma and induces apoptosis by direct effect on mitochondria in neuroectodermal tumors [8]. It also suppresses NF-κB activation induced by carcinogens and inflammatory stimuli [9]. Herein we present the investigation of anticancer effects of Taraxastane, a plant based triterpene, against human cervical cancer. The study also focused on exploring the underlying mechanism of its action.

Methods

MTT assay

Both normal HCvEPc cells and cancer DoTc2 cell lines were plated at a density of 5×10^3 cells/well in 96-well plates for 24 h. The cells were then treated with Taraxastane at 0, 1.25, 2.5, 5, 10, 20, 40, 80, 160 and 320 μM concentrations for 24h. Post treatment, the cells were washed with phosphate buffered saline (PBS) and incubated at 37°C for 4h with 100 ml of 0.25 mg/ml MTT. This was followed by the removal of MTT solution and incubation with 200 ml Tris-DMSO solution for 30 min for solubilizing the MTT-formazan product. Absorbance was measured at 570 nm using a Kinetic Microplate reader (Molecular Devices, Sunnyvale, CA, USA). The experiments were performed at least in triplicate.

Determination of ROS and mitochondrial membrane potential

The plating of DoTc2 cells was done at a density of 2×10^5 cells/well in 6-well plates. The plates were kept for 24 h and then treated with 0, 10, 20 and 40μM Taraxastane for 24h at 37°C in 5% CO_2 and 95% air. This was followed by collection of cells from all treatments which were washed 3 times with PBS and re-suspended in 500 μl of DCFH-DA (10 μM) for estimation of ROS count and $DiOC_6$ (1 μmol/l) for MMP and incubated at 37°C in the dark for 30 min. Flow cytometry was used to analyze the samples instantly as previously described [10].

Cell cycle analysis by flow cytometry

Untreated DoTc2 cells and cells treated with 10, 20 and 40μM of Taraxastane were collected and fixed using 70% ethanol at 4°C overnight. The cells were then centrifuged and resuspended in PBS containing 0.1% Triton X-100, 200μg/ml RNase A and 50μg/ml propidium iodide (PI), before their dark incubation at 37°C for 30 min. The analysis of cell cycle phase distribution was performed through flow cytometry, at $>10^4$ cell count for each sample.

Wound healing assay for cell migration

Briefly, DoTc2 cells were cultured in 6-well plates for 24 h. This was followed by 0 and 20 μM Taraxastane treatment. Thereafter, a scratch line was made on the cells using a 200 μl sterile pipette tip. The plates were incubated at 37°C in 5% CO_2 and 95% air. An inverted microscope system was used to observe the wound healing at 0 and 24 h.

Transwell assay for cell invasion

The invasion of DoTc2 cells and cells treated with 10, 20 and 40μM Taraxastane was determined using transwell chamber assay. In brief, the cells were seeded at the density of 1×10^4 cells in 250 μl serum free Dulbecco's modified Eagles' medium (DMEM) and placed on the upper chambers of the transwell. The chambers were placed into 24-well plates having 600 μl RPMI containing 20% fetal bovine serum (FBS) and incubated at 37°C for 48 h. The non-invaded cells were removed by swabbing from the upper surface of the insert. The invaded cells onto the lower surface were fixed with methanol and stained with 0.5% crystal violet for 1h and washed with PBS. The cells were counted using an upright light microscope (Olympus, Japan) with 100× magnification. Cell counting and average calculation was done from as many as ten random field views.

Western blotting

DoTc2 cells and cells treated with 10, 20 and 40μM Taraxastane were cultured for 24h. Afterwards, the cells were harvested by centrifugation and washed with ice-cold PBS. The cell pellets were re-suspended in RIPA lysis buffer. Bradford assay was employed to determine the protein content of cell lysates. Thirty μg of protein were loaded on SDS-PAGE from each sample before being shifted to polyvinyl fluoride membrane. The membranes were then treated with tris buffered saline (TBS) and exposed to primary antibodies at 4°C. This was followed by treatment of appropriate secondary antibodies. The proteins of interest were visualized using enhanced chemiluminescence reagent.

DAPI staining

The DoTc2 cells were placed in 6-well plates at cell density of 1×10^5. The cells were then treated with 0, 10, 20 and 40 μM of Taraxastane for 24h at 37°C. Next, 25 μl of cell culture were put onto glass slides and stained with DAPI. The slides were then cover-slipped and examined for cell apoptosis under fluorescence microscope.

Statistics

The experiments were performed in triplicate. Values are shown as mean±SD. Statistical analyses were performed

using Students *t*-test with GraphPad prism7 software. Values of p<0.05 were considered significantly different.

Results

Taraxastane inhibits the proliferation of cervical cancer cells

Taraxastane (Figure 1A) was seen to dramatically inhibit the proliferation of DoTc2 cervical cancer cells in a dose-dependent manner. The cell viability decreased with increase in Taraxastane concentration with IC_{50} of 20μM for the molecule. Interestingly, Taraxastane was seen to have lower effect on the proliferation of HCvEPc normal cervical cells as evidenced from higher IC_{50} of around 80μM for normal cells (Figure 1B).

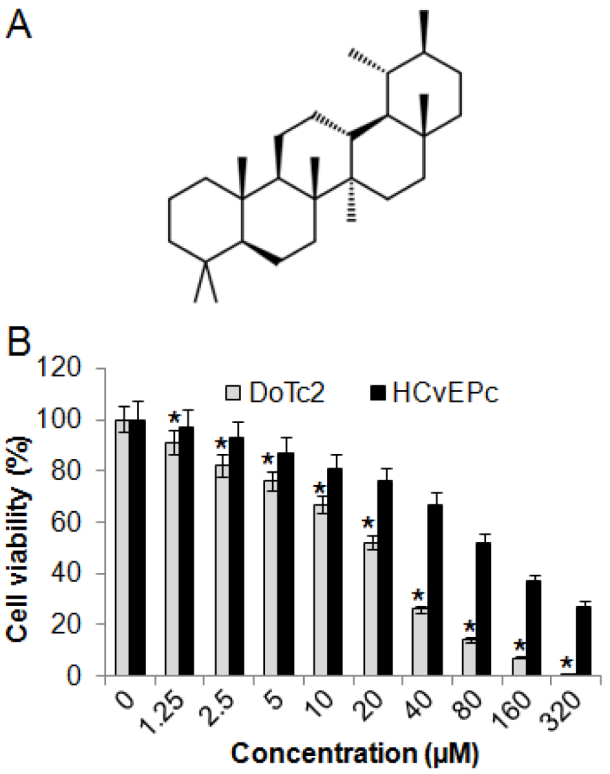

Figure 1. Taraxastane inhibits the growth of cervical cancer cells. **A:** Structure of Taraxastane and **B:** Cell viability assay showing the impact of Taraxastane on the viability of the DoTc2 and HCvEPc cells was dose-dependent. The experiments were performed in triplicate and expressed as mean ± SD (*p<0.05).

Taraxastane inhibits the migration and invasion of DoTc2 cells

The anticancer effects of Taraxastane on DoTc2 cervical cancer cells were also evident by its ability to inhibit the migration and invasion of cancer cells. Wound healing assay was performed to analyze the effect of Taraxastane on the migration of cancer cells and a dramatic reduction in cell migration was noted at 20μM treatment after 24h (Figure 2). Taraxastane was also seen to significantly reduce the invasion of DoTc2 cells in transwell assay. The percentage of invading cells decreased in a dose-dependent manner from 100 to 60, 40 and 27 for 10, 20 and 40μM concentrations, respectively (Figure 3).

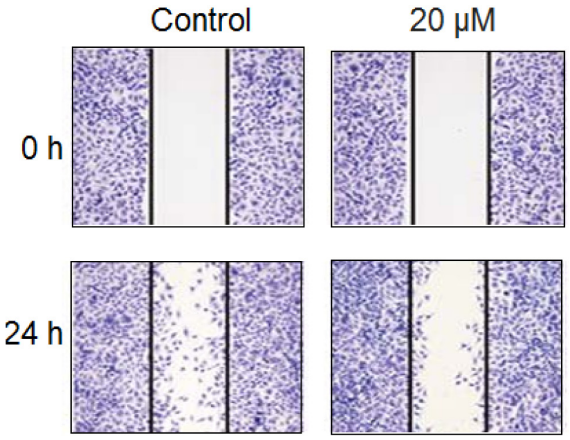

Figure 2. Wound healing assay showing that Taraxastane inhibits the migration of the human DoTc2 cervical cancer cells. The experiments were performed in triplicate.

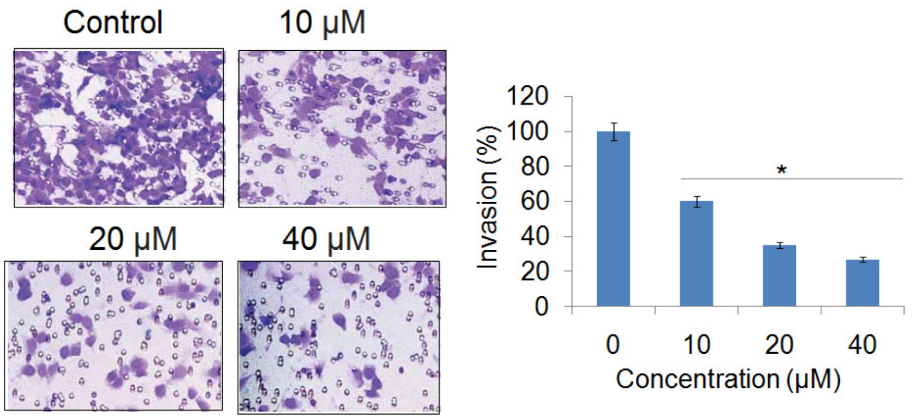

Figure 3. Transwell assay showing that Taraxastane inhibits the invasion of the human DoTc2 cervical cancer cells. The experiments were performed in triplicate and expressed as mean ± SD (*p<0.05).

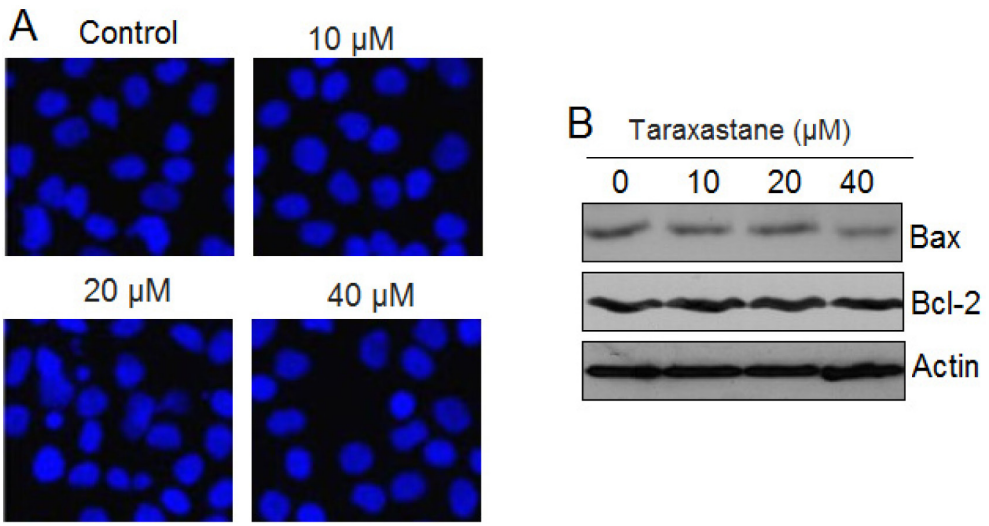

Figure 4. A: DAPI staining showing the induction of apoptosis in the human DoTc2 cervical cancer cells. **B:** Western blotting showing increase in Bax and decrease in Bcl-2 expression upon Taraxastane treatment. The experiments were performed in triplicate.

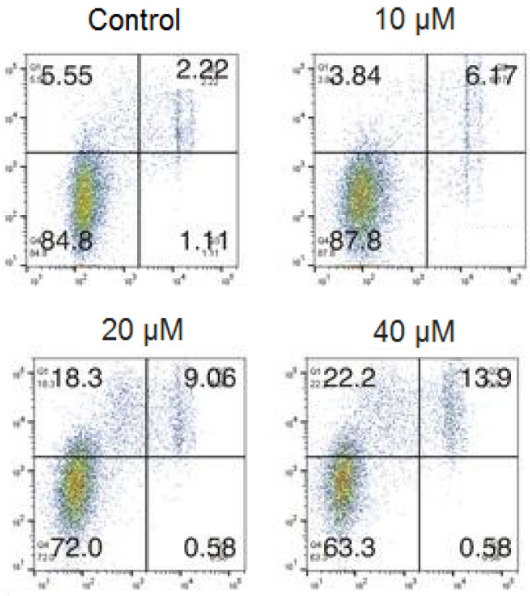

Figure 5. Annexin V/PI staining showing dose-dependent increase in the apoptotic DoTc2 cells upon Taraxastane treatment. The experiments were performed in triplicate.

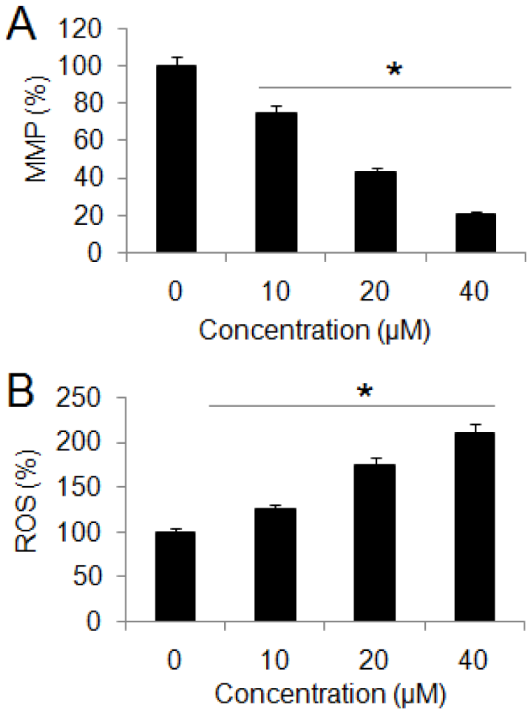

Figure 6. Effect of Taraxastane on the **(A)** MMP and **(B)** ROS levels in DoTc2 cells showing concentration-dependent decrease in MMP and increase in ROS levels of the DoTc2 cells. The experiments were performed in triplicate and expressed as mean ± SD (*p<0.05).

Taraxastane induces necrosis in DoTc2

DAPI staining of DoTc2 cells treated with 0, 10, 20 and 40μM Taraxastane showed that the molecule didn't induce apoptosis of cancer cells (Figure 4A). This was also confirmed by western blotting of Bax and Bcl-2 proteins whose expression remained unchanged under different concentration of Taraxastene (Figure 4B). However, the flow cytometric analysis of DoTc2 cancer cells treated with 0, 10, 20 and 40μM Taraxastane showed that the anticancer effects of the molecule were mediated mainly through cell necrosis. The percentage of necrotic cells was 2.22, 6.17, 9.06 and 13.9 for untreated at 10, 20 and 40μM concentrations, respectively (Figure 5).

Taraxastane increases ROS and decreases MMP in DoTC2 cells

When DoTc2 cells were treated with Taraxastane they showed higher accumulation of ROS and reduction in MMP. The parameters showed dose-

dependent variation. MMP percentage was seen to decrease from 100 to 77, 40 and 20 under 10, 20 and 40µM Taraxastane treatments, respectively (Figure 6A). ROS increased 1.2, 1.75 and > 2-folds when DoTc2 cells were treated with 10, 20 and 40µM Taraxastane, repectively (Figure 6B).

Taraxasatane induces G2/M cell cycle arrest

In order to investigate the effect of Taraxastane on DoTc2 cell cycle, DoTc2 cells were treated with different concentrations of Taraxastane and analyzed for cell cycle phase distribution using flow cytometry. There was significant increase in the percentage of G2/M phase cells and the increase of percentage was found to be concentration-dependent. The percentage of G2/M phase cells was 9, 25, 30 and 58 at 0, 10, 20 and 40µM Taraxastane concentrations, respectively (Figure 7A). Western blot analysis was also performed to examine the effects of Taraxastane on cell cycle related protein, cyclin D1 which showed that the molecule inhibited the expression of cyclin D1 in a dose-dependent manner (Figure 7B).

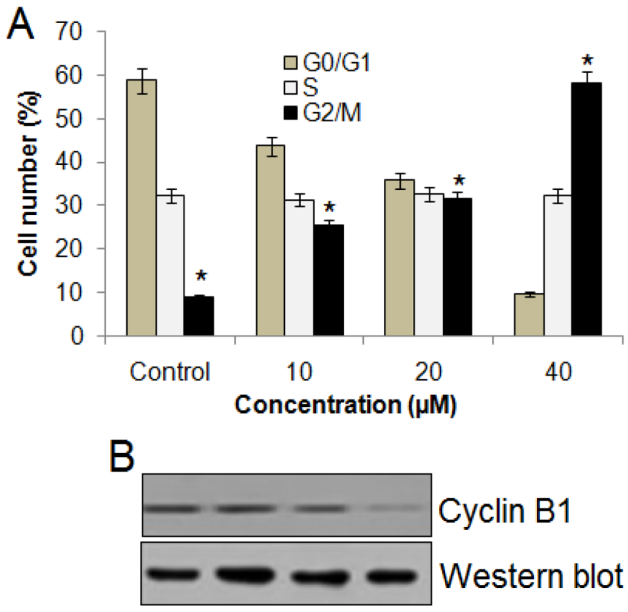

Figure 7. A: Cell cycle analysis showing induction of G2/M cell cycle arrest upon Taraxastane treatment of the DoTc2 cells. **B:** Western blotting analysis showing suppression of cyclin B1 in DoTc2 cells upon Taraxastane treatment. The experiments were performed in triplicate and expressed as mean ± SD (*p<0.05).

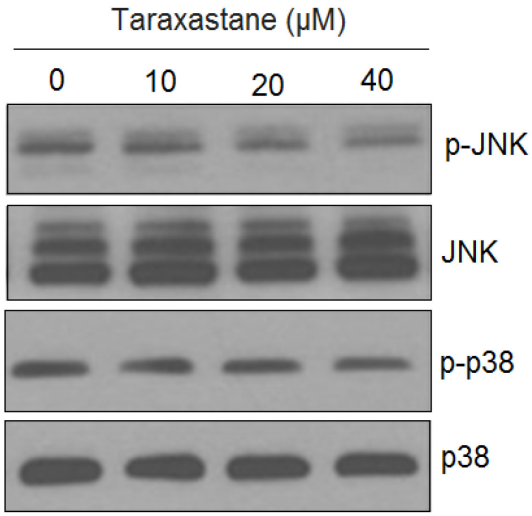

Figure 8. Western blotting analysis showing Taraxastane treatment blocks the JNK/MAPK signaling pathway. The experiments were performed in triplicate.

Taraxastane blocks the JNK/MAPK signalling pathway

Western blot analysis was performed to investigate the effect of Taraxastane on JNK/MAPK signaling pathway of DoTc2 cancer cells. The results suggested that Taraxastane works by blocking the JNK/MAPK signaling pathway by preventing the phosphorylation of crucial signaling components, namely JNK and p38. There was no effect on the concentration of non-phosphorylated JNK and p38 proteins. Also, the inhibition of phosphorylation occurred in a concentration-dependent manner (Figure 8).

Discussion

Plant constitutes vital natural reservoirs for a vast number of natural products of prime economic and pharmacological importance [11]. A number of studies have focused on various plant natural products for their evaluation against different types of human cancers [12-14]. Herein we investigated the anticancer effects of a plant base triterpene, Taraxastane, against human cervical cancer. Human cervical cancer is considered as one of the lethal cancers with mortality rate as high as 90% in low income countries [15]. Plant Taraxastane-type triterpenes have already been reported to have anticancer effects by researchers [16]. The results of present study revealed Taraxastane as a potent anticancer lead molecule. The molecule was found to remarkably reduce the proliferation of DoTc2 cancer cell line. However, its antiproliferative effects were interestingly low on normal HCvEPc. Furthermore, Taraxastane exerted its anticancer effects in a concentration-dependent fashion via mainly necrotic-like cell death, but no correlation of Taraxastane treatment was found for cell apoptosis. Similar sort of anticancer effect was proved for Caffeic acid n-butyl ester by Zhang et al in 2017 [17]. Further, the western blot analysis showed that Bax/Bcl-2 ratio remained unchanged, indicative that Taraxastane is having a negligible effect on

apoptosis of cancer cells. Again, Taraxastane treatment resulted in dose-dependent increase of ROS. It is now well established that enhancement of ROS production is associated with cell death [18]. The Taraxastane treatment also resulted in reduction of MMP. The loss of MMP along with increase in ROS production leads to failure of apoptosome formation and results in cell necrosis [19]. Western blotting experiments showed that the anticancer effects of Taraxastane were exerted via the blockage of JNK/MAPK signaling pathway. The molecule blocked the signaling cascade by inhibiting the phosphorylation of Jun N-terminal kinase (JNK) and p38 mitogen signaling components, which are prime to cancer development [20]. Drastic enrichment of cell cycle progression is one of the important features of human cancers and an external treatment which blocks the cell cycle progression may prove an effective strategy to combat cancer development. The results of this study suggested that Taraxastane promoted the cell cycle arrest of DoTc2 cancer cells by blocking the progression of cell division at G2/M phase. This is also true for other plant natural products as reported by Pandey et al in 2019 [21]. Taraxastane is seen to inhibit cancer cell migration and invasion, which are vital to metastasis and cancer progression. The significance of the current study is that it might pave way for exploration, usage and development of more effective anticancer agents employing triterpenes in general and Taraxastane type natural products in particular as lead molecules against human cervical cancer.

Conclusion

The findings of the present study indicate that Taraxastane affects the progression of cervical cancer by inducing ROS-mediated reduction of MMP, leading to necrotic-like cell death of DoTc2 cancer cells. The study paves way for further evaluation of Taraxastane in cancer therapy.

Conflict of interests

The authors declare no conflict of interests.

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