

# Effects of hirsuteine on MDA-MB-453 breast cancer cell proliferation

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Received July 20, 2022; Accepted October 21, 2022

DOI: 10.3892/ol.2022.13590

**Abstract.** Hirsuteine is extracted from *Uncaria rhynchophylla*, the bark of which has traditionally been used to treat hypertension, cancer, convulsions, hemorrhage, auto-immune disorders, and other ailments. The anticancer properties of hirsuteine are of significant importance to the research community; however, its underlying mechanism of action is not well understood. The aim of the present study was to examine the antiproliferative ability of hirsuteine using human breast cancer MDA-MB-453 cells and to determine the underlying molecular mechanism involved in its therapeutic efficacy. The effects of hirsuteine on cell viability were determined using CCK-8 and colony formation assays, while apoptosis was assessed using flow cytometry. Cell cycle distribution was assessed using flow cytometry, and apoptotic cell quantification was performed using via Annexin V-FITC/PI staining and flow cytometry. Reverse transcription-quantitative PCR and western blotting were used to assess the expression of cell cycle progression and apoptosis associated genes and proteins. MDA-MB-453 cell proliferation was significantly reduced by hirsuteine in a concentration and time-dependent manner. Hirsuteine-treated cells exhibited G2/M phase arrest, as evidenced by the increase in G2/M phase cells and a decrease in the G0/G1 phase cells, and this was related to cyclin B1 and CDK1 downregulation. Furthermore, hirsuteine accelerated MDA-MB-453 cell apoptosis by downregulating Bcl-2 while upregulating cytoplasmic cytochrome c, Bax, Apaf1, cleaved caspase-3, and cleaved caspase-9 levels, which together drove apoptotic cell death. Thus, hirsuteine suppressed MDA-MB-453 cancer cell proliferation by inducing cell cycle arrest and promoting apoptosis.

## Introduction

The 2020 Global Cancer Statistics report published by the International Agency for Research on Cancer showed that in 2020, female breast cancer (BC) was the leading cause of cancer-associated death in women worldwide, with 2,261,419 new cases, accounting for 24.5% of all new cancer cases in women. Additionally, there were 684,996 deaths, accounting for 15.5% of all cancer-associated deaths in 2020 (1). Metastatic BC remains a major contributor to cancer-associated mortality in women. Multiple reports have shown that 20-30% of BC patients have distant metastases when first diagnosed (2-4). Gene expression profiling has had a considerable impact on our understanding of breast cancer biology. In the past 15 years, technological progress has revealed the emergence of at least five different molecular subtypes (Luminal A, Luminal B, HER-2 enriched, Basal-like and Claudin low) and normal breast like groups, which are based on gene expression clustering (5). According to the biological characteristics of the different subtypes, different targeted therapeutics are used in the clinic. For example, trastuzumab, an anti-HER2 drug, has significantly changed the therapeutic field of BC management in the past 20 years (6,7). However, its concomitant cardiotoxicity and the chances of acquired drug resistance remain significant challenges that need to be overcome. Trastuzumab is a targeted drug for the treatment of triple-negative BC, although it is associated with adverse reactions such as neutropenia, a decrease in white blood cell count and anaemia (8-11). Cell cycle arrest has been used in the field of BC treatment with CDK inhibitors such as Palbociclib, Ribociclib, and Abemaciclib (12). Since the approval of Palbociclib by the FDA in 2015, CDK4/6 inhibitors have become a first-line treatment option for patients with metastatic hormone receptor-positive (HR<sup>+</sup>)/HER2-negative (HER-2<sup>-</sup>) BC (13,14).

The use of CDK4/6 inhibitors alone or in combination with other drugs has also become a research hotspot in recent years (15-18). Despite significant progress in the field of chemotherapy-based treatments for BC, as well as anti-angiogenic therapy, immunotherapy, targeted therapy, and other emerging therapies, the clinical effects of these approaches remain unsatisfactory, and the effective rate of immunotherapy is generally low (19,20). Therefore, there is an urgent need to identify novel treatment strategies for BC.

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**Key words:** hirsuteine, breast cancer, cell cycle arrest, MDA-MB-453 cells, apoptosis

Phytochemicals are promising sources for the development of novel cancer therapeutics, due to their potential efficiency and low toxicity profiles (21,22), and they have generally been shown to be promising for the development of novel agents in the management of numerous diseases (23,24). Plant phytochemicals represent an exciting opportunity for improving an individual's general health through a balanced and appropriate diet, and have also been considered suitable options for identifying novel therapeutic agents.

Traditional Chinese Medicines (TCMs) have been used to treat numerous human diseases in China for thousands of years (25,26), and have served as a remarkable source for drug discovery (27). In the present study, the effect of hirsuteine (Fig. 1), an active compound extracted from the traditional well-known Chinese herb *Uncaria rhynchophylla*, on BC was assessed *in vivo*. Several studies have shown that hirsuteine possesses a number of therapeutically relevant properties, especially for treating central nervous system and cardiovascular disorders, namely, hypertension, epilepsy, dizziness, convulsion, preeclampsia, and tremor, amongst others, initially confirming the efficacy and safety (28-30).

However, the antiproliferative activity and the underlying mechanisms by which hirsuteine reduces cancer development and progression have not been determined. Cancer progression is dependent on its unique ability to escape programmed cell death (31). Apoptosis is usually initiated by the death receptor (DR) (extrinsic) or mitochondrial (intrinsic) axis, and it functions to eliminate injured cells to maintain homeostasis (32). In both axes, active caspase 3 and 7 cleave poly-ADP ribose polymerase 1 (PARP1) following DNA degeneration (33). The intrinsic network is mitochondrial-based apoptosis, which involves cytochrome c release and activation of caspase-9, which in turn activates caspase-3. The extrinsic network is dependent on DR stimulation, which in turn induces the FAS-related death domain (FADD) and generates the death-induced signal complex, which regulates downstream caspases-8, -7, -6, and -3 (34,35).

Research on the active ingredients of botanicals have received increasing attention. Our research group has primarily studied the TCM *Uncaria rhynchophylla*, and hirsuteine is an alkaloid extracted from *Uncaria rhynchophylla*, which has a significant inhibitory effect on several cancer cell lines (36,37). Our previous study demonstrated that hirsuteine is cytotoxic to numerous tumor cells *in vitro* (38). Nevertheless, despite its valuable properties, little is known regarding its antitumor capacity and the possible mechanism of hirsuteine in BC. Therefore, determining the underlying mechanisms by which hirsuteine affects BC cancer development and progression was assessed in the present study.

The aim of the present study was to examine the possible anticancer effects of hirsuteine and the molecular mechanisms underlying its therapeutic efficacy. In particular, hirsuteine-mediated regulation of MDA-MB-453 cell apoptosis was assessed. The distribution of cells in the cell cycle and apoptosis induction were examined in MDA-MB-453 cells.

## Materials and methods

**Chemicals and reagents.** Hirsuteine (ST17300105; 5 mg/dose; purity  $\geq 98\%$ ) was acquired from Shanghai

Standard Technology Co., Ltd. The CCK-8 Detection kit, BSA, and Annexin V-FITC Detection kit were acquired from Beyotime Institute of Biotechnology. B-cell lymphoma-2 (Bcl-2; ab32124; dilution, 1:1,000), Bcl-2-associated X protein (Bax; ab32503; dilution, 1:1,000), Cyclin B1 (ab32053; dilution, 1:1,000), CDK1 (ab133327; dilution, 1:1,000), Apaf1 (ab234436; dilution, 1:1,000), cytochrome C (ab133504; dilution, 1:1,000), cleaved-caspase3 (ab32042; dilution, 1:1,000), cleaved-caspase9 (ab2324; dilution, 1:1,000), cleaved-PARP1 (ab32064; dilution, 1:1,000),  $\beta$ -actin (ab8227; dilution, 1:5,000) antibodies, goat anti-rabbit IgG secondary antibody (ab6721; dilution, 1:20,000) were purchased from Abcam. Other reagents were analytical reagent grade and from commercial sources.

**Cell culture.** Human BC MDA-MB-453, MDA-MB-231, and MCF-7 cells were acquired from American Type Culture Collection. Cells were cultured using RPMI 1640 or DMEM supplemented with 10% FBS, penicillin (100 U/ml), and streptomycin (100  $\mu\text{g}/\text{ml}$ ). Normal human breast cells Hs 578Bst, human normal lung epithelial cell BEAS-2B, and normal human hepatocyte THLE-2 cells were obtained from Nanjing KeyGen Biotech Co., Ltd. and cultured in DMEM supplemented with 10% FBS, penicillin (100 U/ml), and streptomycin (100  $\mu\text{g}/\text{ml}$ ) in a humidified incubator supplied with 5%  $\text{CO}_2$  at 37°C.

**CCK-8 assay.** Cells were plated in 96-well culture plates ( $1 \times 10^4$  cells/well) overnight and then treated with a range of hirsuteine concentrations (0, 2.5, 5, 10, 20, 40, or 80  $\mu\text{M}$ ) for 24, 48 and 72 h, as described previously (38). For Hs 578Bst, THLE-2, and BEAS-2B cells, assay protocols were as above, with cells being treated for 48 h with various concentrations of hirsuteine (0, 2.5, 5, 10, 20, 40, or 80  $\mu\text{M}$ ) prior to addition of the CCK-8 reagent. The 50% growth inhibition ( $\text{IC}_{50}$ ) value was determined using a hirsuteine survival concentration curve. All experiments were performed at least three times.

**Colony formation assay.** Cells were plated in 6-well culture plates at  $2.5 \times 10^3$  cells per well for 24 h. Cells were then exposed to varying hirsuteine concentrations (0, 5, 10, or 25  $\mu\text{M}$ ) for 48 h, after which the hirsuteine-containing media was removed, and fresh media was added. The cells were then cultured for 14 days in supplemented media. Finally, cells were fixed in methanol at room temperature for 15 min, dyed with 1% crystal violet solution at room temperature for 10 min, washed with PBS three times, dried in the room and observed under a microscope, with images captured. The number of colonies consisting of  $\geq 50$  cells was counted.

**Apoptosis staining.** Cells were exposed to varying concentrations of hirsuteine (0, 5, 10, or 25  $\mu\text{M}$ ) for 48 h and apoptosis was measured as described previously (38).

**Cell cycle analysis.** Briefly, a six-well plate was seeded with  $3 \times 10^5$  cells per well. After incubation, the media was replaced with supplemented media containing a range of hirsuteine concentrations (0, 5, 10, or 25  $\mu\text{M}$ ) for an additional 48 h. Cells were then detached using 0.25% trypsin, and centrifuged at 4°C for 5 min at 300 x g, washed once with PBS, and fixed

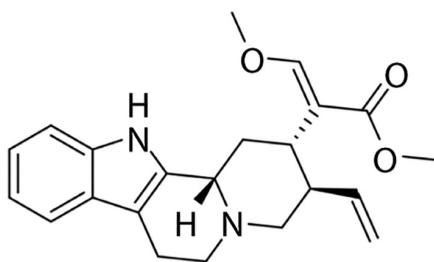


Figure 1. Chemical structure of hirsuteine (MW=366.45).

overnight at 4°C with 70% chilled ethanol. Subsequently, the cells were centrifuged again as above, fully suspending the cells after treating with 100  $\mu$ l RNase A (50  $\mu$ g/ml) at 37°C in a water bath for 30 min, prior to a 30 min staining at 4°C in the dark with 400  $\mu$ l PI (50  $\mu$ g/ml). All samples were evaluated using a FACScan flow cytometer (BD Biosciences). Data were analyzed using CellQuest Pro software, version 5.1 (BD Biosciences). All experiments were performed three times independently.

**Western blotting.** Following 48 h of treatment with hirsuteine (0, 5, 10, or 25  $\mu$ M), chilled RIPA buffer was used for cell lysis for 30 min. Extracted proteins were resolved and transferred as described previously (38). The membranes were blocked for 1 h at 37°C with 5% milk in Tris-buffered saline (TBS) containing 0.05% Tween-20 (TBST) and then incubated with cleaved caspase-3, cleaved caspase-9, cleaved CARP, Bax, Bcl-2, Apaf1, cytochrome c, CDK1, Cyclin B1 and  $\beta$ -actin (Abcam) antibodies at 4°C overnight. Membranes were then washed using TBST and incubated with the goat anti-rabbit IgG secondary antibody (Abcam) at room temperature for 2 h. Immunoblotted proteins were analyzed with the ChemiDoc XRS imaging system and QuantityOne software (Version 4.6.9; Bio-Rad Laboratories, Inc.).

**Reverse transcription-quantitative PCR (RT-qPCR).** Cells were treated with different concentrations of hirsuteine for 48 h. Total RNA was isolated from cells using TRIzol<sup>®</sup> reagent followed by reverse transcription to cDNA using a QuantiTect Reverse Transcription kit, according to the manufacturer's protocol. Subsequently, qPCR was performed using ChamQ<sup>™</sup> SYBR qPCR MasterMix in triplicate according to the manufacturer's protocol. Each reaction was conducted in duplicate using the following thermocycling conditions: Initial denaturation for 10 min at 95°C, followed by 40 cycles of denaturation for 30 sec at 95°C, annealing for 30 sec at 60°C and extension for 20 sec at 72°C. The sequences of the primers were: Bax forward, 5'-AAGAAGCTGAGCGAGTGTCT-3' and reverse, 5'-GTTCTGATCAGTTCCGGCAC-3'; Bcl-2 forward, 5'-GCC TTCTTTGAGTTCGGTGG-3' and reverse, 5'-GAAATCAAA CAGAGGCCGCA-3'; caspase-3 forward, 5'-ACTGGACTG TGGCATGAGA-3' and reverse, 5'-GCACAAAGCGAC TGGATGAA-3'; caspase-9 forward, 5'-ACATGCTGGCTT CGTTTCTG-3' and reverse, 5'-TCTCAAGAGCACCGACAT CA-3'; and GAPDH forward, 5'-TCAAGAAGGTGGTGA AGCAGG-3' and reverse, 5'-TCAAAGGTGGAGGAGTGG GT-3'. GAPDH was used as the loading control. Expression was quantified using the  $2^{-\Delta\Delta C_q}$  method (39).

**Statistical analysis.** Data are presented as the mean  $\pm$  SD from three independent repeats. Statistical comparisons were performed by one-way analysis of variance, followed by Bonferroni's test.  $P < 0.05$  was considered to indicate a statistically significant difference. Data were analyzed using GraphPad Prism version 6.0 (GraphPad Software Inc.).

## Results

**Cytotoxicity of hirsuteine against human BC MDA-MB-453 cells.** Hirsuteine-mediated regulation of MDA-MB-453, MDA-MB-231, and MCF-7 cell viability was assessed using a CCK-8 assay (Fig. 2A). Hirsuteine lowered MDA-MB-453 cell viability in a concentration and time-dependent manner (Fig. 2B). Next, we explored the impact of hirsuteine on the proliferation of Hs 578Bst, BEAS-2B, and human hepatocyte THLE-2 cells using a CCK-8 assay. The results showed that the cell viability after hirsuteine treatment in normal cells was  $>80\%$ , which was considered non-toxic for the non-cancerous cell lines after 48 and 72 h of treatment at doses up to and including 80  $\mu$ M (Fig. 2C and D). As indicated in Fig. 2E and F, hirsuteine markedly reduced colony formation in a concentration-dependent manner. Based on the results, hirsuteine inhibited MDA-MB-453 cell proliferation and lowered MDA-MB-453 colony formation activity, whilst not exerting a notable effect on healthy human cell lines at the same doses.

**Hirsuteine inhibits cell cycle progression.** Cell-cycle distribution was assessed using flow cytometry. Apoptosis can be characterized by DNA fragmentation and damage. There are multiple phases and subphases in the cell cycle, namely, G0/G1 (DNA pre-synthesis and stationary); S (DNA synthesis); and G2/M (DNA post-synthesis and mitosis) (40–42). To elucidate whether hirsuteine altered cell cycle distribution, the number of cells in each phase of the cell cycle was evaluated following hirsuteine treatment. Based on the results, the proportion of cells in the G0/G1 phase was reduced from  $56.1 \pm 3.4$  to  $11.5 \pm 1.3\%$ , while the proportion of cells in the G2/M phase increased from  $5.9 \pm 2.6$  to  $42.9 \pm 3.9\%$  significantly, and the proportion of cells in the S phase increased from  $38.1 \pm 1.2$  to  $45.5 \pm 2.9\%$  following hirsuteine treatment (Fig. 3A). The proportion of cells in each phase of the cycle were compared (Fig. 3B). To determine the potential mechanism by which cell cycle arrest was induced by hirsuteine, western blotting was used to determine the expression levels of cell cycle-associated proteins in hirsuteine treated MDA-MB-453 cells. A marked decrease was observed in CDK1 and Cyclin B1 protein levels, which may underlie the G2/M phase arrest induced by hirsuteine (Fig. 3C and D). Thus, hirsuteine promoted the progression of BC cells from the S phase to the G2/M phase, prior to cell cycle arrest in G2/M phase, resulting in a reduction in proliferative ability and cell viability.

**Hirsuteine induces MDA-MB-453 cell apoptosis.** Hirsuteine-treated (0, 5, 10, and 25  $\mu$ M) MDA-MB-453 cell apoptosis was quantified using flow cytometry using Annexin V labeling and PI exclusion staining. A four-quadrant schematic was used to classify the following cell states: Mechanical, necrotic, normal, early apoptotic, and late

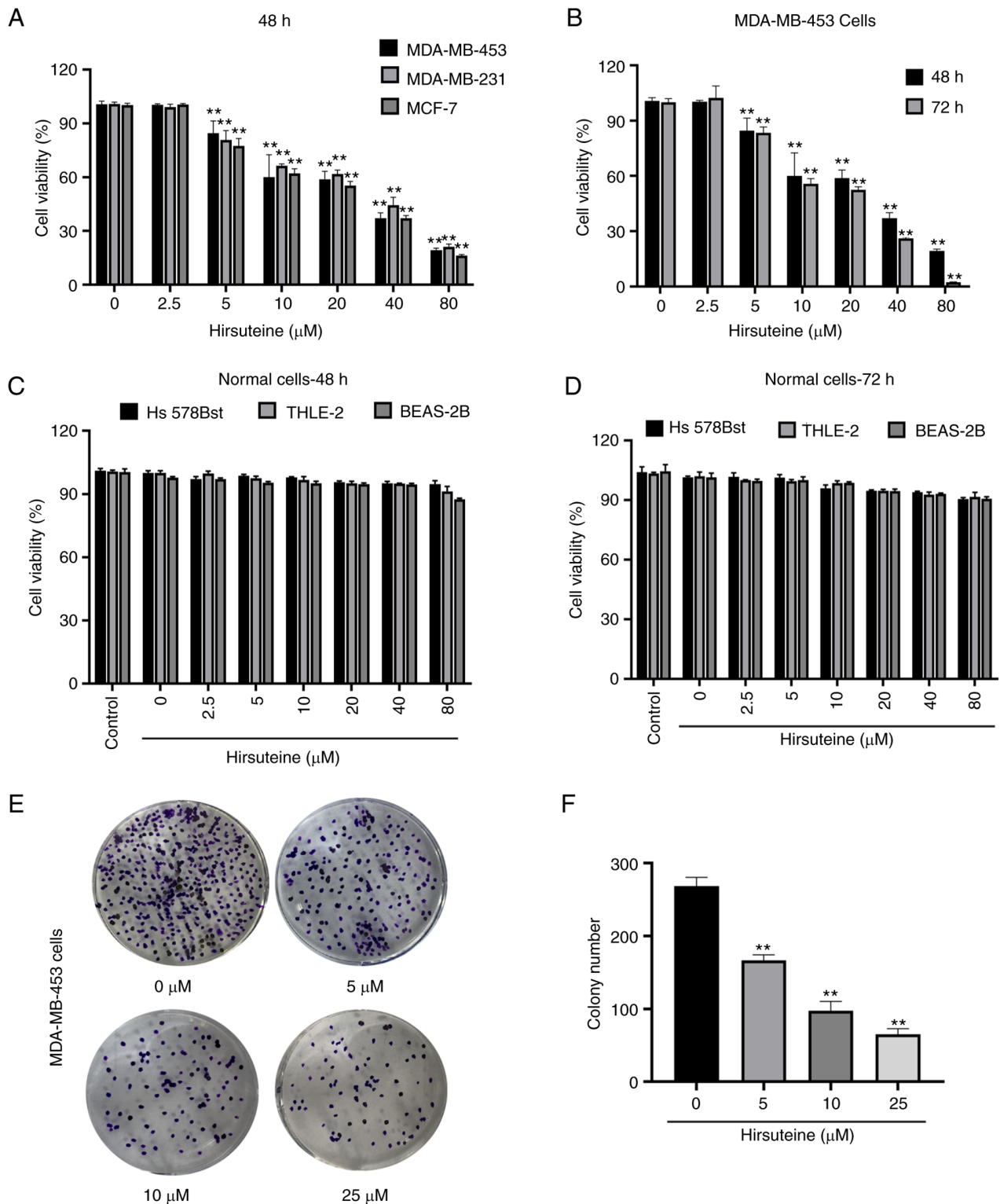


Figure 2. Hirsuteine suppresses MDA-MB-453 cell proliferation and colony formation. (A) The proliferative capacity was assessed in MDA-MB-453, MDA-MB-231, and MCF-7 cells following treatment with hirsuteine for 48 h using CCK-8 assays. (B) Time-dependent MDA-MB-453 cell proliferation curve following treatment with varying hirsuteine concentrations. (C and D) Normal Hs 578Bst, THLE-2, and BEAS-2B were exposed for 48 and 72 h to a series of hirsuteine concentrations, prior to CCK-8 analysis. (E and F) Colony formation assays using MDA-MB-453 cells following treatment with hirsuteine. Data are presented as the mean  $\pm$  SD of three repeats. \*\* $P < 0.01$ .

apoptotic. Relative to the control cells, the quantity of healthy cells decreased from  $97.7 \pm 0.2$  to  $77.5 \pm 0.4\%$ , and the quantity of early and late apoptotic cells increased from  $1.4 \pm 0.2$  to  $22.1 \pm 0.4\%$  with increasing hirsuteine concentrations (Fig. 4A). We also recorded the number of apoptotic cells in the control

and treated groups (Fig. 4B). Thus, hirsuteine suppressed MDA-MB-453 cell proliferation whilst inducing apoptosis.

*Hirsuteine regulates apoptosis-related proteins.* Bax, Bcl-2, cleaved caspase-3, and cleaved caspase-9 expression levels



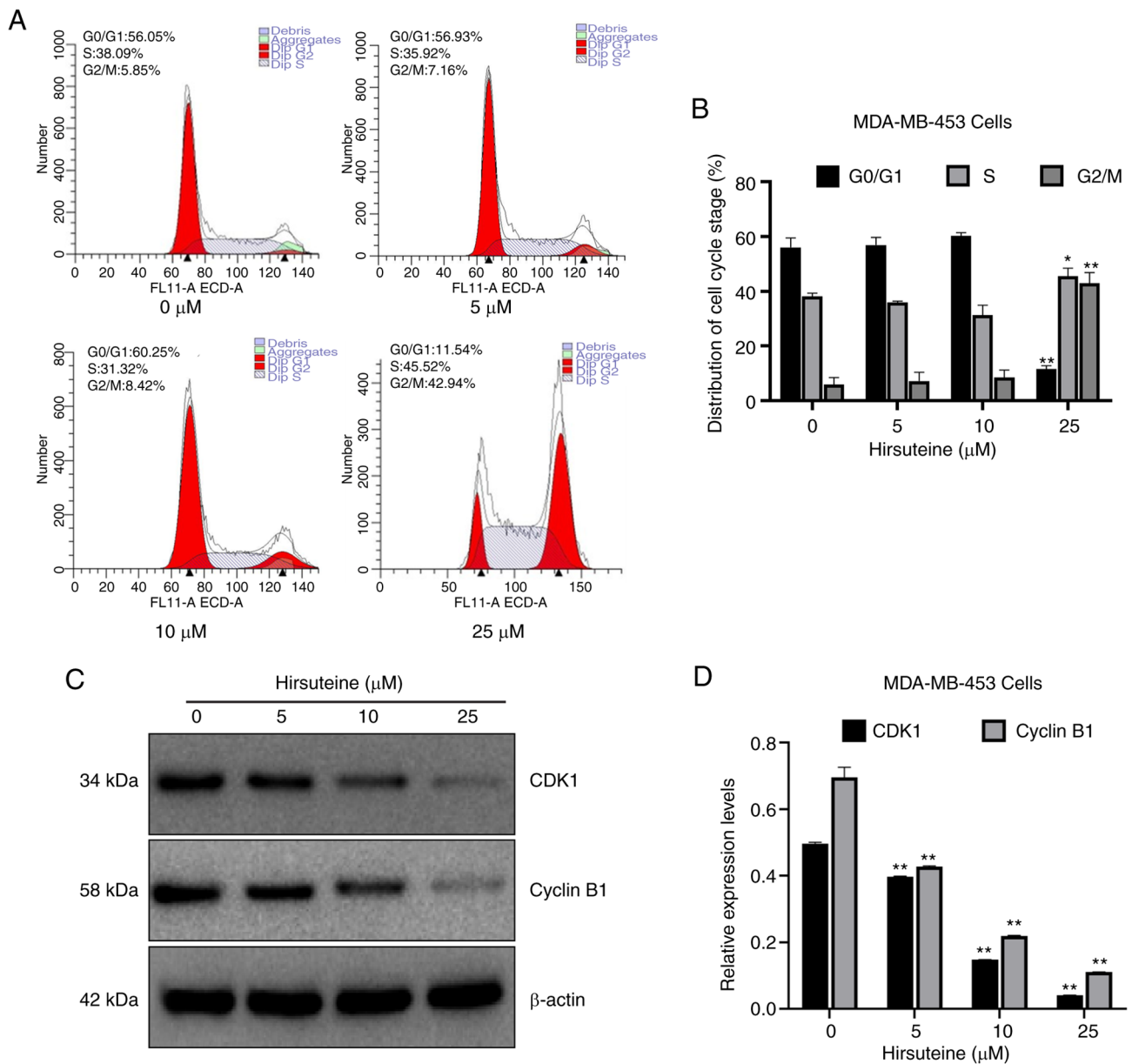


Figure 3. Hirsuteine-mediated regulation of the cell cycle distribution. (A) Flow cytometry analysis of cell cycle phases in MDA-MB-453 cells following treatment with hirsuteine for 48 h (0, 5, 10, or 25  $\mu$ M). (B) Percentage of cells in each phase of the cell cycle. (C) Western blot analysis of Cyclin B1 and CDK1 protein expression in hirsuteine-treated MDA-MB-453 cells. (D) Quantification and analysis of Cyclin B1 and CDK1 protein expression levels. Data are presented as the mean  $\pm$  SD of three repeats. \* $P$ <0.05, \*\* $P$ <0.01.

were assessed following 48 h of treatment with hirsuteine using qPCR and western blotting. Following treatment with different hirsuteine concentrations for 48 h, the RT-qPCR results showed that in the MDA-MB-453 cells, Bax, cleaved-caspase-3, and cleaved caspase-9 mRNA levels were promoted and Bcl-2 expression was decreased (Fig. 5A-D), resulting in an increase in pro-apoptotic/anti-apoptotic protein ratio. After 48 h of treatment with different doses of hirsuteine, the western blot results showed that hirsuteine increased the levels of cleaved caspase-3, cleaved caspase-9, and cleaved-PARP levels in the MDA-MB-453 cells (Fig. 5E). Additionally, densitometry analysis of the protein expression levels was performed, and the results are shown in Fig. 5F-H. These results showed that hirsuteine induced apoptosis in MDA-MB-453 cells via the intrinsic apoptotic pathway.

*Hirsuteine induces apoptosis in MDA-MB-453 cells via the Bcl-2/Bax axis.* The Bcl-2 family members induce apoptosis via the mitochondrial network (43). To examine the hirsuteine-mediated regulation of cell apoptosis, we assessed the expression levels of several apoptotic proteins. After 48 h of treatment, with hirsuteine (0, 5, 10, or 25  $\mu$ M), increased expression of the proapoptotic Bax, Apaf-1, and cytoplasmic cytochrome-c levels, and reduced levels of Bcl-2 were observed, resulting in an imbalance in the Bax/Bcl-2 ratio relative to control cells in a dose-dependent manner (Fig. 6A). Furthermore, densitometry analysis of the protein expression levels was performed, and the results are shown in Fig. 6B-E. Together, these results demonstrated that hirsuteine activated the Bcl-2/Bax signaling pathway, resulting in apoptosis in MDA-MB-453 cells.

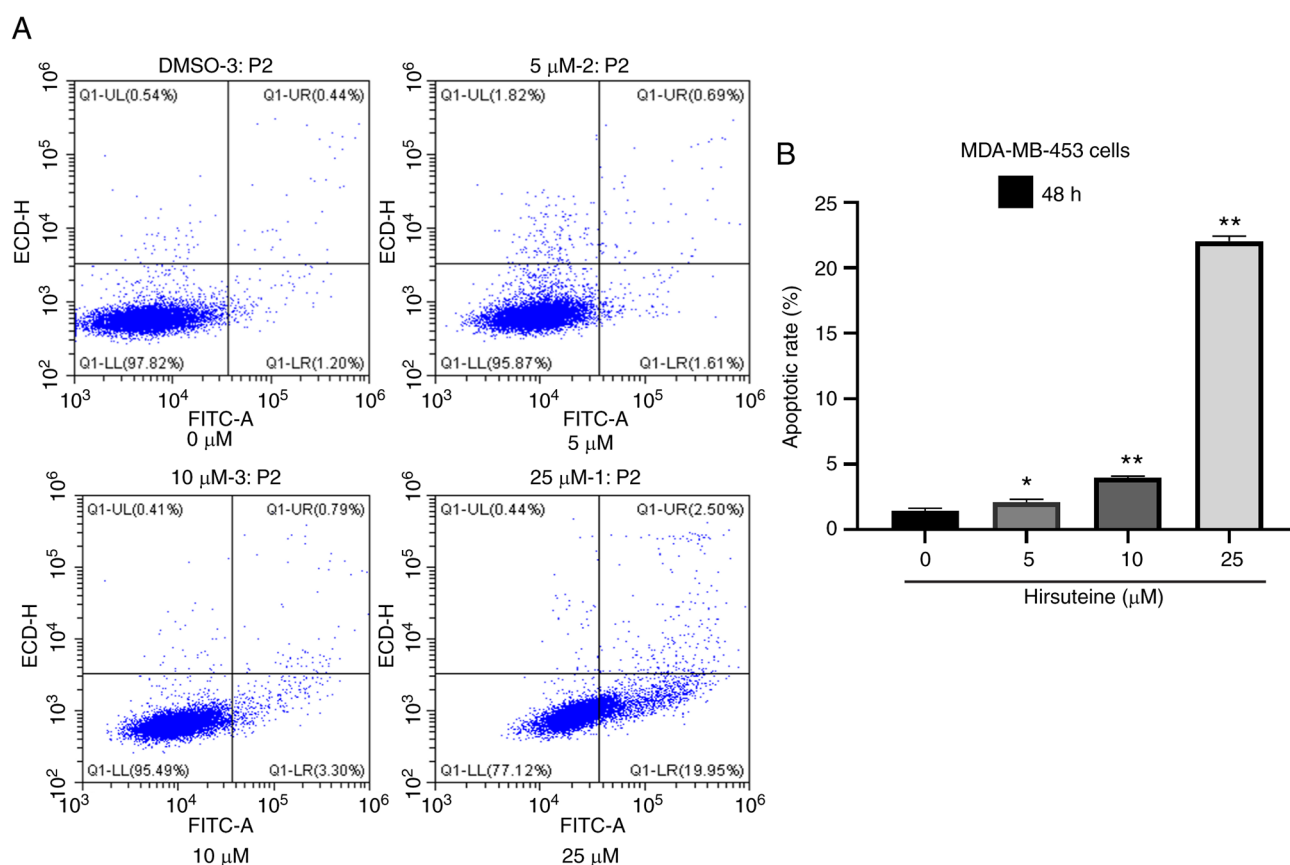


Figure 4. Hirsuteine-mediated regulation of MDA-MB-453 cell apoptosis. (A) Annexin V- FITC/PI staining was used to stain cells using flow cytometry following treatment of MDA-MB-453 cells with hirsuteine for 48 h (0, 5, 10, or 25  $\mu$ M). (B) Quantification of apoptosis of the MDA-MB-453 cells. Data are presented as the mean  $\pm$  SD of three repeats. \* $P$ <0.05, \*\* $P$ <0.01.

## Discussion

At present, chemotherapy is the primary method of treatment of BC, and it can lead to several side effects (44). The common adverse reactions of traditional chemotherapeutic drugs include gastrointestinal reactions, bone marrow toxicity, hepatorenal toxicity, cardiotoxicity, neurotoxicity, and systemic reactions (45). The adverse reactions caused by immune checkpoint inhibitors differ from traditional chemotherapy-related adverse effects. Immune-related adverse events (IRAEs) caused by immune checkpoint inhibitors usually include endocrine dysfunction, skin toxicity, gastrointestinal adverse reactions, and a small number of cases of liver toxicity (46,47). The incidence of serious IRAEs is low, but if it exceeds expectations and cannot be appropriately handled, it may endanger a patient's life (46). Therefore, increasing attention is being paid to the discovery of safe and more effective novel compounds for the treatment of patients with BC.

Phytochemicals are promising compounds that have been used for several years to develop cancer medications owing to their potential efficacy and reduced toxicity profiles (21,22). Multiple reports have suggested that a majority of *Uncaria rhynchophylla*-mediated biological properties can be attributed to the alkaloid constituents (48,49). Hirsuteine may serve as a novel and specific SPHK1 inhibitor, exerting anti-leukemic activity by inhibiting the SPHK1/S1P/S1PR1 and BCR-ABL/PI3K/Akt pathways in CML cells (36).

Similarly, we previously reported that the oxindole alkaloid purified from *Uncaria rhynchophylla* could effectively suppress the survival of Jurkat Clone E6-1 cells (T-cell leukemia cells) (38).

In the present study, CCK-8 assays of the effect of hirsuteine on MDA-MB-231 and MCF-7 breast cancer cells were used to determine the safe concentration range of hirsuteine. For all subsequent experiments, 25  $\mu$ M was used as the upper limit as the inhibition rate of hirsuteine on the proliferation of MDA-MB-453 cells was about 45% at this concentration. However, in future studies, 50%  $IC_{50}$  dose,  $IC_{50}$  dose, and 2x  $IC_{50}$  dose will be used. In this study, it was shown that hirsuteine exhibited cytotoxicity against cancer cells in a concentration-dependent manner (MDA-MB-453) without affecting normal cells (Hs 578Bst, THLE-2 and BEAS-2B), which corroborates earlier investigations which showed that *Uncaria rhynchophylla* inhibited the proliferation of numerous tumor cell lines (50-52). The results of flow cytometry also revealed the effectiveness of hirsuteine on human MDA-MB-453 BC cells, indicating the underlying mechanisms by which hirsuteine exerts its anti-cancer-specific effects.

Cell cycle arrest is a promising approach for inhibiting tumor development, particularly since multiple studies demonstrated notable cell cycle dysregulation in several types of cancer (53-56). Based on the results of the present study, hirsuteine-treated MDA-MB-453 cells exhibited  $G_2/M$  phase cell cycle arrest and displayed enhanced cleaved caspase 3

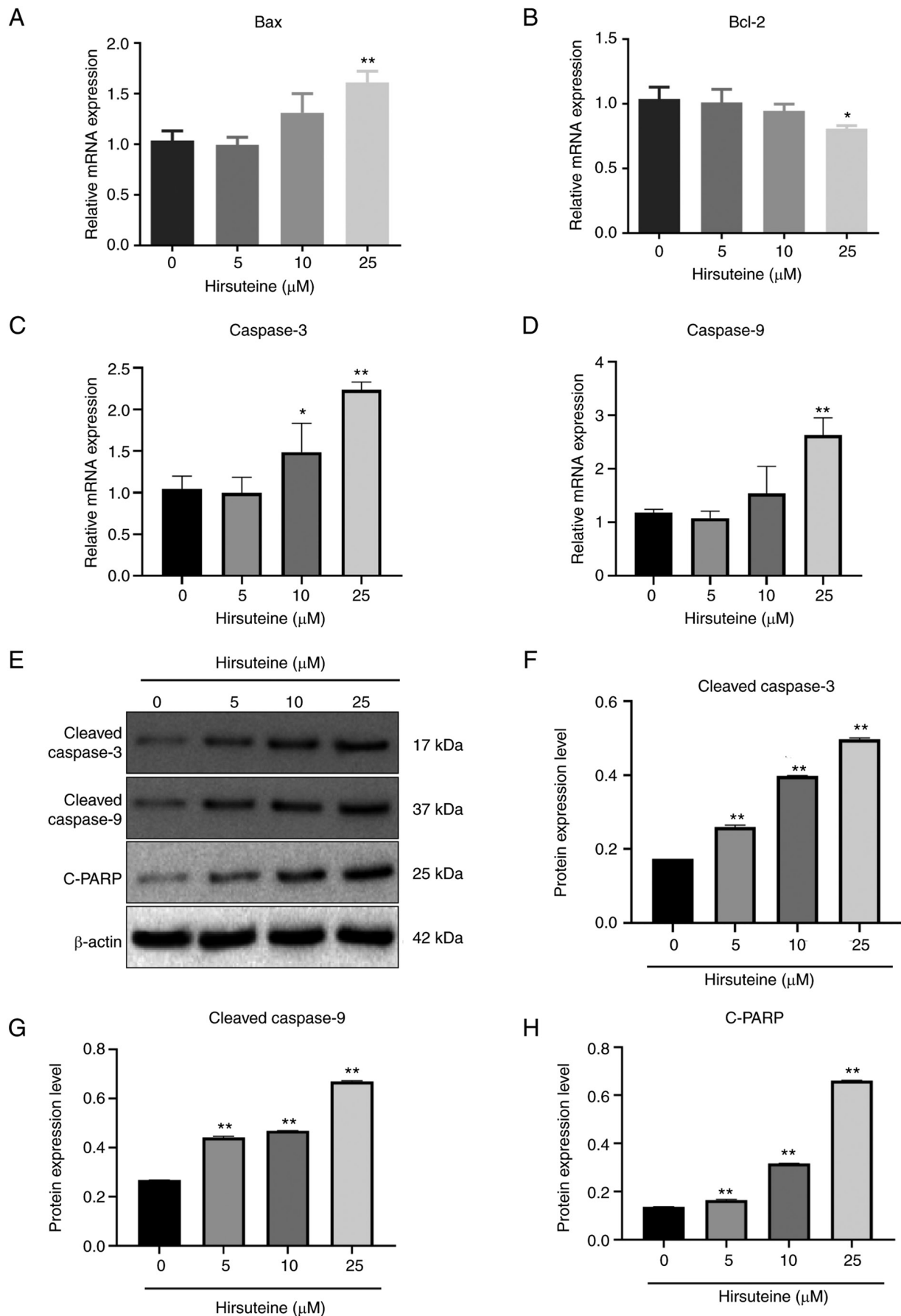


Figure 5. Alterations in the expression levels of apoptosis-related proteins and mRNA in hirsuteine-treated MDA-MB-453 cells. Histogram of the mRNA levels of (A) Bax, (B) Bcl-2, (C) caspase 3, and (D) caspase 9 in MDA-MB-453 cells. (E) The relative protein levels of cleaved caspase-3, cleaved caspase-9, and cleaved-PARP in MDA-MB-453 cells treated with hirsuteine. (F-H) Protein expression levels were normalized to the control group. Data are presented as the mean  $\pm$  SD of three repeats. \* $P<0.05$ , \*\* $P<0.01$ .

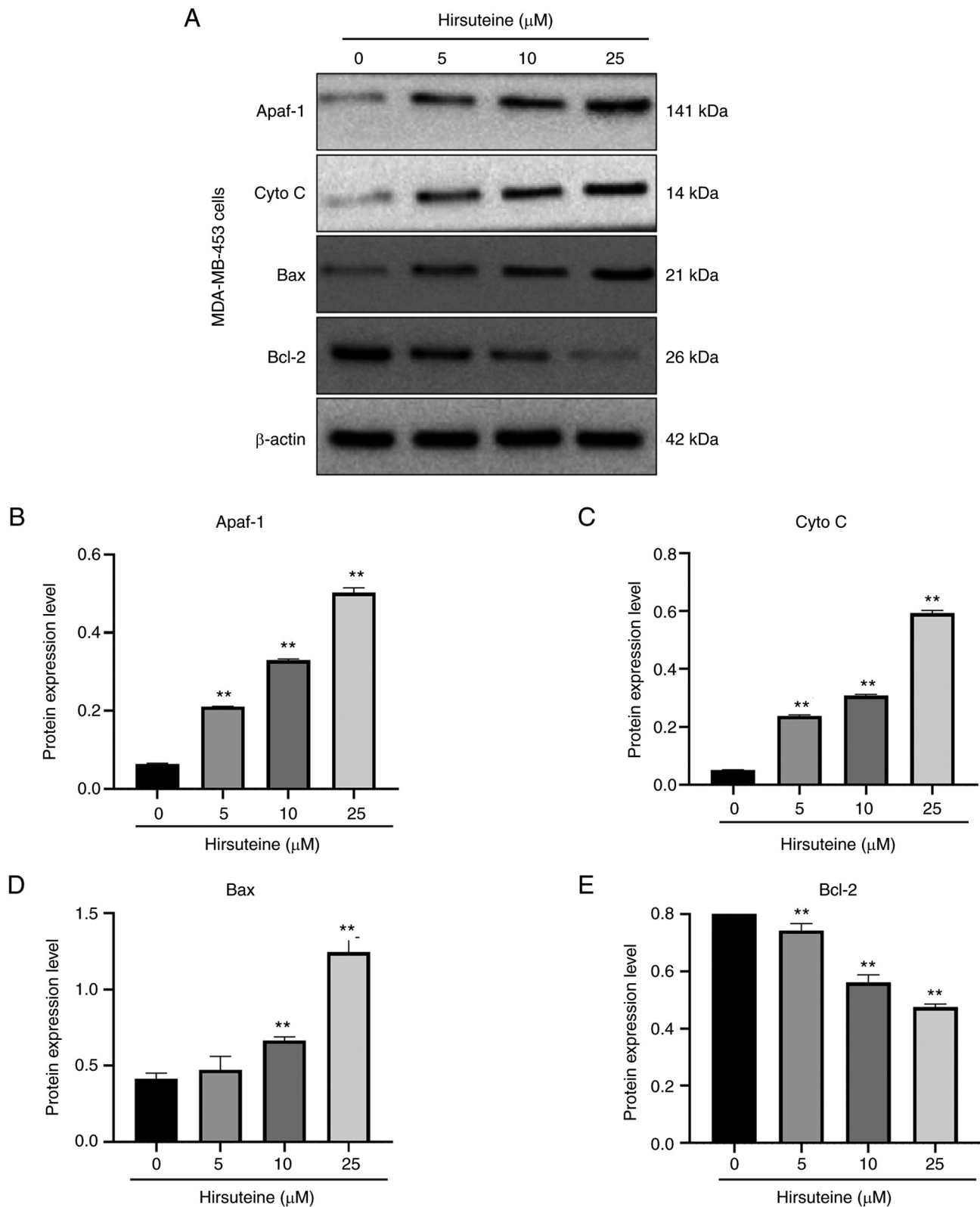


Figure 6. Hirsuteine-mediated regulation of the Bcl-2/Bax axis. (A) Western blot analysis of the Apaf1, cytoplasmic cytochrome-c, Bax, and Bcl-2 levels in MDA-MB-453 cells exposed to varying hirsuteine concentrations (0, 5, 10, or 25  $\mu\text{M}$ ) for 48 h. (B) Apaf1, (C) cytochrome C, (D) Bax, and (E) Bcl-2 protein levels following normalization to Actin. Data are presented as the mean  $\pm$  SD of three repeats. \*\* $P < 0.01$ .

levels, indicative of an increase in the cell cycle following hirsuteine treatment. It was previously reported that CDK1 modulates cell cycle progression via interaction with cyclins B1 and A2 (57).

There is increasing evidence that numerous viral proteins can cause host  $G_2/M$  cell cycle arrest. The  $G_2/M$  arrest induced by some viral proteins is linked to the inhibition of cyclin B1-CDK1 kinase activity, inactivating the cyclin B1-CDK1



complex, which in turn prevents premature entry into the M phase (58,59). Hence, Cyclin B1 is responsible for entry into the M phase (60,61). Upon entry into prophase, Cyclin B1-CDK1 activity gradually increases (62), which activates the Cyclin B1-CDK1 complex, resulting in its translocation to the nucleus to promote cell division (63). Based on the western blot analysis of the aforementioned study, there was a strong association between decreased CDK1 and cyclin B1 levels with suppressed MDA-MB-453 cell proliferation following hirsuteine treatment, which eventually resulted in cell cycle arrest in the G<sub>2</sub>/M phase.

Apoptosis activation occurs in one of two methods: i) the intrinsic axis involves mitochondrial cytochrome c release, which induces various downstream caspases, and ii) the extrinsic axis which involves activation of the Fas death receptor via an external stimulus (64). The intrinsic apoptotic axis does not require the activation of a membrane receptor. In fact, the signal is generated in the mitochondria itself, and this axis is critical for drug-activated apoptosis, whereas the extrinsic apoptotic axis involves membrane-bound death receptors that belong to the TNF gene superfamily (65,66). Caspase-9 (intrinsic axis) and caspase-8 (extrinsic axis) activate caspase-3 (67-70). Caspase-9 initiates the apoptotic process by promoting the formation of the apoptosome complex within the mitochondrial network. Caspase-3 cleaves both PARP and DNA, which are hallmarks of the apoptotic process. Upon release of mitochondrial cytochrome c, it interacts with the adaptor protein Apaf-1 (71). Herein, hirsuteine substantially augmented Bax, PARP1, caspase-9, and caspase-3 expression, indicating that the mitochondria-based apoptotic network contributed to the hirsuteine-driven increase in MDA-MB-453 cell apoptosis. Collectively, the results of the present study showed that the intrinsic apoptotic axis was stimulated by hirsuteine in MDA-MB-453 cells.

In conclusion, this study revealed that hirsuteine strongly suppressed human BC cell development via activation of the caspase-3-based apoptotic network. These results highlight a novel target for the management of BC, as well as a promising agent for further assessment.

## Acknowledgements

Not applicable.

## Funding

No funding was received.

## Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

## Authors' contributions

BY, YY and JM designed the present study. JM and YY performed the experiments. JM and YL analyzed the data. JM drafted the initial manuscript. BY and YY revised the initial manuscript. JM and BY confirm the authenticity of all the raw

data. BY and YY helped guide JM in the whole process. All authors have read and approved the final manuscript.

## Ethics approval and consent to participate

Not applicable.

## Patient consent for publication

Not applicable.

## Competing interests

The authors declare that they have no competing interests.

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