# Naringin induces endoplasmic reticulum stress-mediated apoptosis, inhibits β-catenin pathway and arrests cell cycle in cervical cancer cells

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**Naringin is a promising anticancer bioflavonoid phytochemical, mainly extracted from citrus fruits. This study evaluates the antiproliferative effect and the cell death mechanism induced by naringin on cervical cancer (CC) cells. Our results demonstrated that naringin exerts significant inhibition in cell viability and exhibits $IC_{50}$ value 745, 764, 793 μM against C33A, SiHa, and HeLa cells respectively. Annexin V FITC and immunoblotting analysis reveal significant apoptosis induction in cells exposed to higher doses naringin. Mechanistically, naringin induces endoplasmic reticulum (ER) stress-associated cell killing in CC cells. Naringin increases the protein expression of ER stress sensors, phosphorylates eIF2α by and activates apoptosis-associated protein CHOP and other associated proapoptotic proteins (PARP1 and caspase-3). Intriguingly, pre-treatment with of ER stress inhibitor (salubrinal), reverses the apoptotic effect exerted by naringin. Additionally, the naringin abrogates the β-catenin pathway by decreasing the protein expression as well as phosphorylation of β-catenin (Ser576) and GSK-3β (Ser9) and simultaneously triggers cell cycle arrest at a $G_0/G_1$ phase by increasing the expression of cell cycle checkpoint proteins p21/cip and p27/kip. Naringin induces ER stress-mediated apoptosis and simultaneously abrogates Wnt/β-catenin signaling which eventually triggers the arrest of the cell cycle at a $G_0/G_1$ phase in CC cells.**

**Key words**: Naringin, apoptosis, endoplasmic reticulum stress, cell cycle, cervical cancer

**Abbreviations**: CC, cervical cancer; FITC. Fluoro-isothiocyanate; ER, endoplasmic reticulum; CHOP, C/EBP homologous protein; eIF2α, eukaryotic initiation factor 2 alpha; PARP1, Poly (ADP-ribose) polymerase 1; GSK-3β, glycogen synthase kinase-3 beta; β-catenin, beta-catenin, UPR, unfolded protein response; Bip, binding immunoglobulin protein; PERK, protein kinase R (RKR)- like endoplasmic reticulum kinase; ATF-6, activating transcriptional factor-6, IRE1, inositol requiring enzyme; ATF-4, activating transcriptional factor-4; DMEM, Dulbecco's Minimal Essential Medium; FBS, fetal bovine serum; BPE, bovine pituitary; EGF, epidermal growth factor; MTT, 3-(4, 5-dimethylthiazol-2-y1)-2, 5-diphenyltetrazolium bromide; PI, propidium iodide; PMSF, phenylmethylsulfonyl fluoride; DTT, dithiothreitol; DMSO, dimethyl sulfoxide; HRP, horseradish peroxidase; NP-40, nonidet-P40; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; KCl, potassium chloride; SDS-PAGE, sodium dodecylsulphate-polyacrylamide gel electrophoresis; PVDF, polyvinylidene fluoride; PBS, phosphate buffer saline; TBST, tris-buffered saline tween-20; CDK2, cyclin-dependent kinase-2; CDK4, cyclin-dependent kinase-4.

## INTRODUCTION

One of the commonest cancers, cervical cancer, ranks second leading cause of mortality among women aged 20–39 years (Fidler *et al.*, 2017). Even though chemotherapy is the most standard therapeutic option in cervical carcinogenesis, the 5-year survival rate of patients with cervical cancer is poor and needs to be improved by validating new targets and search for specific antitumor agents with less adverse effects on normal cells (Pfaendler *et al.*, 2016). Therefore, novel therapeutic targets are urgently needed and their elucidation for the betterment of cervical cancer therapeutics.

During the process of tumorigenesis, tumor cells often encounter various stress-related factors such as nutrient deprivation, low oxygen tension (hypoxia), oxidative insults, proteasome dysfunction and associated factors that eventually disturb ER homeostasis (Cubillos-Ruiz *et al.*, 2017; Rah *et al.*, 2016). This disturbance in ER homeostasis interferes in the protein folding process, thereby causes misfolding of proteins which leads to the induction of ER stress (Wang *et al.*, 2014). In response to ER stress, an evolutionarily conserved process known as unfolded protein response (UPR) signaling pathway is activated by disrupting the interaction between chaperone protein (BiP) and ER sensor proteins (PERK, ATF-6, and IRE1), thereby increases and activates chaperone proteins and simultaneously induces the temporary attenuation of protein translation to allow restoration of ER homeostasis (Feldman *et al.*, 2005). However, if the stress is severe, persistent and cannot be restored, UPR activates ER stress-associated cell death pathway by upregulating downstream target CHOP via ATF4 (Jing *et al.*, 2011; Yamaguchi *et al.*, 2004). Recent reports revealed that ER stress performs a crucial role in the cytotoxicity of many natural compounds. These compounds modulate the ER stress pathway in tumor cells and exhibit antitumor activity by inducing ER stress-mediated apoptosis (Kim *et al.*, 2018; Rah *et al.*, 2015).

Naringin, a flavanone glycoside compound, obtained from the Drynaria plant. Commonly used in Chinese herbal medicine, naringin has numerous pharmacological activities (Cavia-Saiz *et al.*, 2010). The various pharmacological activities reported by naringin including antilipidemic (Mallick *et al.*, 2016), anti-atherogenic (Lee *et al.*, 2001), superoxide scavenging and antioxidant (Cavia-Saiz *et al.*, 2010), metal chelating (Jagetia *et al.*, 2004), anticarcinogenic and anti-inflammatory activities (Chtourou *et al.*, 2016). In the recent past, naringin is reported to exhibit antiproliferative activity and promotes cell death in tumor cells to prevent tumor growth. However, the molecular machinery of cell death promoted by naringin

is yet to be elucidated. Therefore, the current study aims to evaluate the anticancer potential of naringin and the mechanism of cell death promoted by naringin in CC cells. We found naringin exhibits strong antiproliferative potential against cervical tumors with less adverse toxicity to normal cells even at higher concentrations. Mechanistically, naringin induces ER stress-associated cell death in CC cells. Additionally, our results revealed that naringin abrogates the β-catenin signaling pathway and triggers the arrest of the cell cycle at a $G_0/G_1$ phase in CC cells. These results revealed the novel insights in understanding the underlying molecular mechanism of naringin and might be a potential drug candidate for future therapeutics in cervical cancer.

## MATERIAL AND METHODS

**Cell culture and treatments**. Human CC cells C33A, SiHa, HeLa and normal cervical cells HCK1T were obtained from American Type Culture Collection (ATCC) and were cultured in the Dulbecco's Minimal Essential Medium (DMEM) (#A4192101; GIBCO) having fetal bovine serum (10%) (FBS; #16000044; Gibco/Invitrogen), penicillin-streptomycin solution (#15140130; Invitrogen), whereas HCK1T normal cervical cells were cultured in Defined Keratinocyte Serum-Free Medium (SFM) (GIBCO) contains extracts of bovine pituitary (BPE) and supplemented with growth factor (EGF) (5 ng/ml) and the humidified incubator with 5% $CO_2$. All the cell lines were free from Mycoplasma contamination.

**Preparation of naringin dilutions**. 20 mg/ml stock solution of naringin was prepared in dimethyl sulfoxide (DMSO) (Sigma, USA). Aliquots of stock solutions were prepared and stored at –20°C. Further, a stock solution was diluted in DMEM to prepare a working solution of desired concentrations between 250 and 2000 μM for the treatment of cultured cells.

**Chemicals, reagents, and antibodies**. Naringin (#71162), 3-(4,5-dimethylthiazol-2-y1)-2, 5-diphenyltetrazolium bromide (MTT; #M5655), propidium iodide (PI; #P4170), phenylmethylsulfonyl fluoride (PMSF; #78830), dithiothreitol (DTT; #10197777001), dimethyl sulfoxide (DMSO; #C6164), Bradford reagent (#B6916), and annexin V and FITC kit for apoptosis detection (#APOAF) and protease inhibitor cocktail (#P8340), were purchased from Sigma. All antibodies were procured from cell signaling technology; β-actin (#3700S; 1:10000 dilution), Poly (ADP-ribose) polymerase (PARP1) (#9532S; 1:1000 dilution), BiP (#3177; 1:1000 dilution), p-eIF2α-ser51 (#3398S; 1:1000 dilution) , CHOP (#5554; 1:1000 dilution), p-PERK (#3179; 1:1000 dilution), Caspase-3 (#9662S; 1:1000 dilution), p-β-catenin-Ser675 (#4176T; 1:1000 dilution), E-cadherin (#14472S; 1:2000 dilution), p-GSK3β-Ser9 (#5558S; 1:1000 dilution), vimentin (#5741; 1:1000 dilution), p-27/Kip1 (#3686; 1:1000 dilution), p-21/Cip1 (#2947; 1:1000 dilution), Cyclin D1 (#55506S; 1:1000 dilution), c-Myc (#18583; 1:1000 dilution), CDK4 (#12790; 1:1000 dilution), and CDK2 (#2546; 1:1000 dilution). Secondary anti-mouse IgG-coupled (#sc-2005; 1:2000 dilution) and antibodies, anti-rabbit coupled (#sc-2357; 1:2000 dilution) with horseradish peroxidase (HRP) were procured from Santa Cruz Biotechnology

**Cell viability assay**. The proliferation of cells was analyzed by MTT assay as per the standard protocol (Rah *et al.,* 2012). Briefly, CC cells (C33A, SiHa, HeLa) along with normal (HCK1T) cells were processed for trypsi-nization and plated at a density $5×10^3$ cells per well of 96-well plate. The cells seeded in triplicates were treated to varying concentrations of naringin (250–2000 μM) and control DMSO for 24 h in an incubator containing 5% $CO_2$. Subsequently, cells were saturated with MTT dye (2.5 mg/ml) for 3 hrs at 37°C. The formazan crystals formed were dissolved in DMSO, mixed properly by vortex and the optical density at 570 nm was recorded using a microplate reader. The cell proliferation percentage was analyzed as the percent cell viability of treated cells compared with the control of DMSO treated cells.

**Detection of apoptosis**. Apoptosis detection and quantification were analyzed by using the Annexin V and FITC kit, as per the manufacturer's protocol and instructions. CC cells were trypsinized and covered at a density of $0.5×10^6$ cells in each well in a 6-well plate and treated to different concentrations (500, 750, 1000 μM) of naringin along with control DMSO. After 24 h, cells were trypsinized or scrapped with a scrapper, processed and incubated in a staining solution containing propidium iodide (PI) and annexin V as per manufacturer's protocol. After completion of the staining process, tumor cells were examined by flow cytometry (BD FACS Aria II, BD Biosciences, San Jose) to quantify the apoptotic population.

**Cell cycle analysis**. CC cells at a density of $0.5×10^6$ were trypsinized and seeded in a 6-well plate and let them adhered to the surface overnight. Cells were exposed to varying concentrations (500, 750, 1000 μM) of naringin along with control DMSO for 24 h. Cells were washed thoroughly with cold PBS and fixed overnight with ice-cold ethanol (70%) at –20°C. Processed cells were incubated at 37°C in RNase solution (100 μg/ml) for 30 minutes and simultaneously incubated at 4°C in PI (50 μg/ml) for another 30 minutes in dim light. The analysis was performed by using the BDTM LSRII system (BD Bioscience, Franklin Lakes, USA)

**Phase-contrast microscopy**. CC cells ($5×10^4$) were cultured on coverslips with varying (500, 750, 1000 μM) concentrations of naringin along with control DMSO for 24 h to analyze the changes in the cellular morphology. Cells were processed for the phase-contrast microscope to analyze the morphological changes in treated cells.

**Immunoblotting.** After overnight plating of $50×10^5$ cells/well in humidified 5% $CO_2$ incubator at 37°C, CC cells were exposed with varying concentrations (500, 750, 1000 μM) of naringin along with DMSO vehicle. After 24 h, harvested cells were washed with ice-cold PBS, and were processed for lysis with lysis buffer containing (protease inhibitor cocktail, DTT 1 mM/L, NP-40 0.3%, HEPES 1 mM/L, sodium orthovanadate 1 mM/L, EDTA 1 mM/L, KCl 60 mM/L, PMSF 0.1 mM/L). The lysis solution obtained from cells was collected and processed at 4°C for centrifugation at a speed of $13000×g$ for 10 min. Supernatants collected from centrifugation in a separate tube were subjected to the Bradford method of protein estimation. The volume of cell lysis solution containing 20 μg of protein was calculated and subjected to SDS-PAGE from each sample treated with different drugs. After resolving properly, SDS-PAGE gel was transferred to the PVDF membrane (Millipore; #IPVH00010), allowed it to incubate with a 5% (w/v) fat-free milk blocking solution containing 0.1% Tween-20 dissolved in PBS (Sigma; #274348). After blocking nonspecific proteins with a blocking solution, the PVDF membrane is incubated and probed with the desired antibodies (1:1000 dilutions) for at least 3 h at room temperature or overnight at 4°C. After incubation, the PVDF membrane is washed with TBST buffer

three times, incubated and probed with species specific secondary antibodies tagged with horseradish peroxidase. Immunosubstance proteins were spotted by enhanced chemiluminescence (ECL) plus (Amersham; #WBKL-SO100).

**Statistical Analysis**. All the experiments were accomplished for at least three independent times. The latest version of software GraphPad Prism was used for statistical analysis of all independent unbiased experiments. The results obtained were denoted as the mean of $\pm$ S.E.M. Entire results were calculated by using the Student's unpaired t-test, wherein a $p$-value of less than 0.05 was reflected significant (*means $p<0.05$. **$p<0.01$, and ***$p<0.001$).

## RESULTS

### Naringin exhibits an antiproliferative effect in CC cells

To evaluate the antiproliferative effect of naringin, CC cells (C33A, SiHa, HeLa) and normal cervical cells HCK1T were cultured in respective medium and exposed with increasing (10–10 000 μM) concentrations of naringin for 24 h. As confirmed (Fig. 1A and 1B), naringin reduces the cell viability of CC cells as the concentrations increase above 100 μM and the effect is significant above 500 μM concentrations of naringin. Using GraphPad prism the $IC_{50}$ concentration of naringin in CC cells is 745, 764, and 793 μM for C33A, SiHa, and HeLa respectively. However, in normal HCK1T cervical cells, $IC_{50}$ of naringin is above 5 000 μM, indicates that naringin specifically kills CC cells and displays less toxicity to normal transformed cervical cells at a concentration where naringin exhibits a strong antiproliferative effect in CC cells.

### Naringin promotes apoptosis in CC cells

To confirm the antiproliferative effect exhibited by naringin, we perform apoptosis assay after treating CC cells with varying concentrations (500, 750, 1000 μM) of naringin along with a control DMSO and staurosporine (25 nM) as a positive control. As shown in (Fig. 2A and 2B) 30.1% population of HeLa cells were observed to undergoes apoptosis which is significant when compared to the apoptotic cell percentage of control DMSO (1.1%) and positive control staurosporine (35.9%). To support the above result, we perform immunoblotting after treating HeLa cells with varying (500, 750,

1000 μM) concentrations of naringin for 24 h to evaluate the expression of proapoptotic proteins. Our immunoblotting results revealed that the cleaved band of poly ADP-ribose polymerase 1 (PARP1) and caspase-3 were observed at 1 000 μM of naringin treated HeLa cells when compared to DMSO control and staurosporine as a positive control (Fig. 2C). However, phase-contrast microscopy showed (Fig. 2D) that morphological changes take place in treated cells and a significant number of floating dead cells were found when HeLa cells were exposed to higher concentrations of naringin, compared to control DMSO and positive control staurosporine. These results indicate that naringin exhibits a strong antiproliferative effect by inducing apoptosis in CC cells.

### Naringin induces ER stress in CC cells

Recent shreds of evidence report that natural compounds obtained from plant sources induce tumor cell death by inducing ER stress (Quan *et al.*, 2010). Therefore, we sought to examine whether naringin could also induce ER stress-mediated cell death in CC cells. After treating cervical malignant cells with increasing concentrations of naringin (500, 750, 1000 μM) for 24 h. Immunoblotting was performed to analyze the expression of ER stress sensors. As shown in (Fig. 3A and 3B) there is a profound expression of stress sensor markers (BiP, CHOP) and simultaneously increases the phosphorylation of p-eIF2α, p-PERK at higher concentration (1000 μM) of naringin. Further our results showed that prominent cleaved products of caspase-3 in cell lysates treated with higher concentrations of naringin indicate the activation of programmed cell death type I pathway in CC cells when exposed to higher concentrations of naringin. To confirm whether the naringin induced apoptosis is dependent on ER stress, we design experiment and treat cervical cells with salubrinal (an ER stress Inhibitor) before naringin treatment for 3 h. Intriguingly, immunoblotting results revealed a drastic reduction in the expression of ER stress markers in cervical cell lysates exposed to higher concentrations of naringin. Subsequently, we didn't observe cleaved products of PARP1 and caspase-3 in the cell lysates when probed with the PARP1 and caspase-3 antibody respectively. However, quantification of apoptotic cells (Fig. 3B) showed that the significant decrease in the apoptotic cell population of naringin treated CC cells which were earlier pre-treated with salubrinal when compared to cells treated with naringin only. This indicates that the induc-
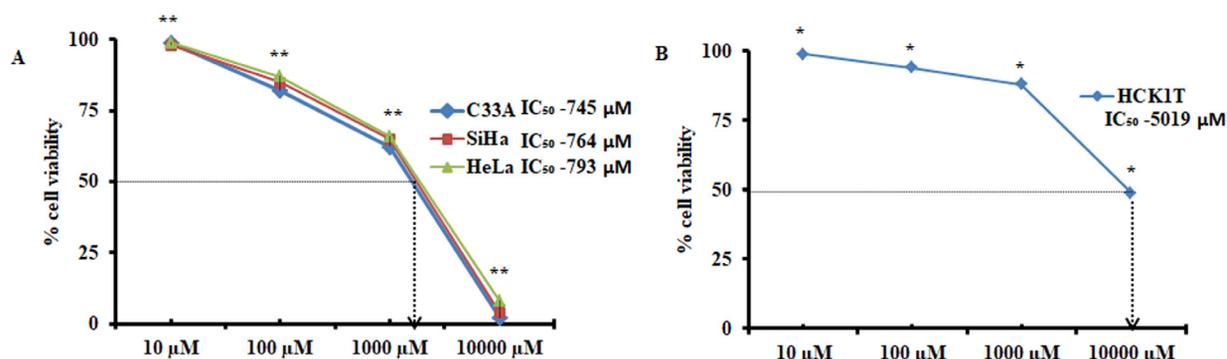


**Figure 1. Naringin prevents cell viability in CC cells.**
(**A**) Influence of naringin on the cell proliferation of CC cells (C33A, SiHa, and HeLa) determined by MTT assay. (**B**) Influence of naringin on the cell proliferation of normal cervical cells (HCK1T) determined by MTT assay. The data denotes the mean value ±S.E. of three independent unbiased experiments *p<0.05, **p<0.01
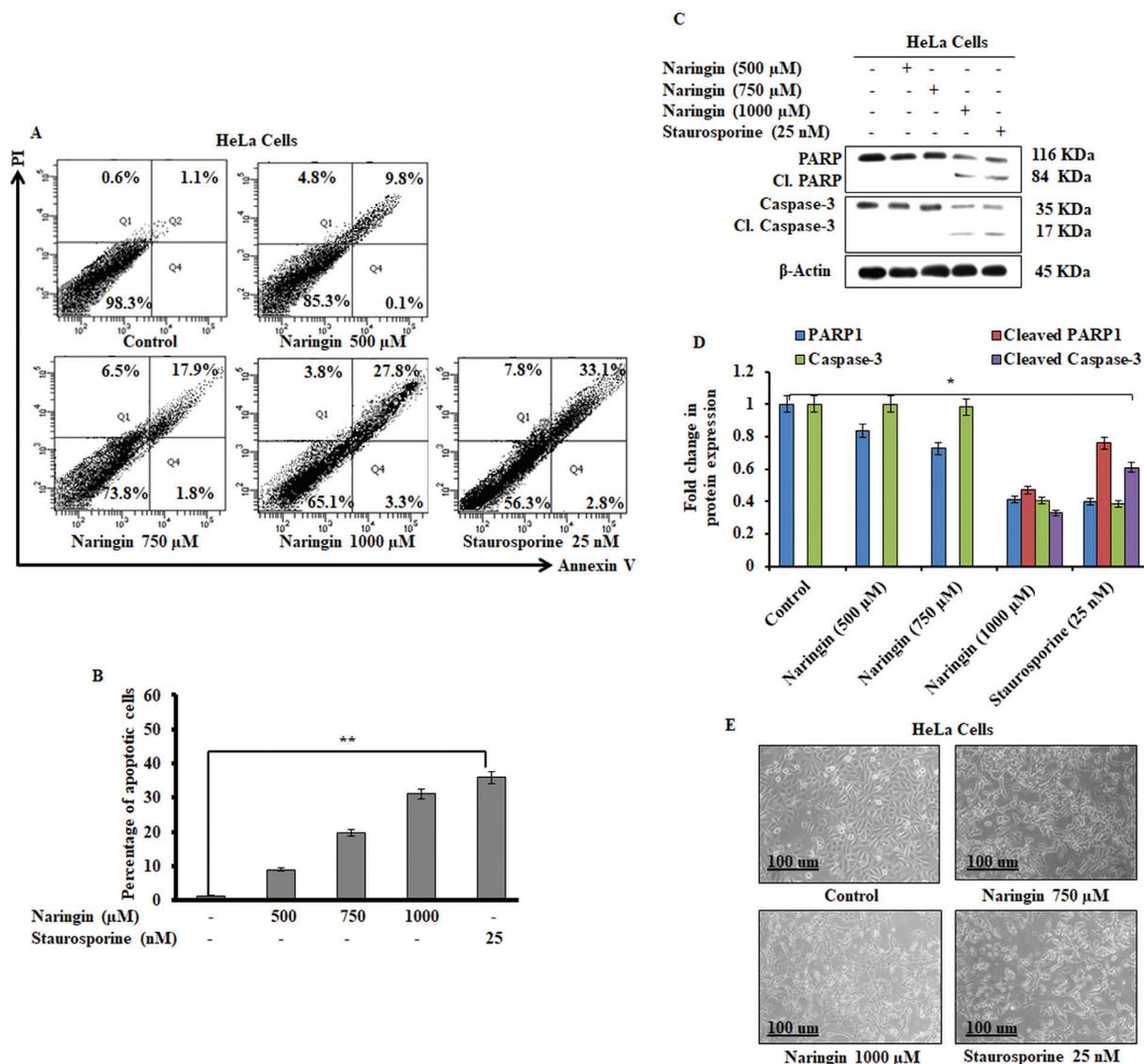
**Figure 2. Naringin induces apoptosis in CC cells.**
(**A**) CC cells (HeLa) were treated with varying (500, 750, 1000 µM) concentrations of naringin along with staurosporine (25 nM) as positive control and DMSO as a control for 24 h and then exposed to staining solution of Annexin V FITC and propidium iodide and analyzed by FACS. (**B**) Bar diagram showing quantification of Annexin V FITC positive cells analyzed by FACS. (**C**) Immunoblotting analysis showing prominent PARP and caspase-3 cleavage in lane 4 and 5 where cells were exposed to naringin and staurosporine treatment and internal control β-actin. (**D**) Densitometry analysis represented by histogram shows the fold change in protein expression after normalized with β-actin. (**E**) Representative micrograph images by phase-contrast microscopy of CC cells (HeLa) exposed to indicate doses of naringin and positive control staurosporine. Scale bar: 100 µm; 20x. The data denotes the mean value ±S.E. of three independent unbiased experiments *$p<0.05$, **$p<0.01$

tion of apoptosis in CC cells is dependent on ER stress-induced by naringin.

### Naringin inhibits β-catenin signaling pathway and triggers cell cycle arrests at a $G_0/G_1$ phase in CC cells

Due to the upregulation of the β-catenin pathway in CC cells (Liang *et al.,* 2016; Rampias *et al.,* 2010; Sun *et al.,* 2017), we sought to examine the effect of naringin in the β-catenin signaling pathway. Our immunoblotting results demonstrate the reduction in the phosphorylation status of β-catenin (Ser675) and GSK3β (Ser9) in CC cells (Fig. 4A). Additionally, we observed the upregulation of E-cadherin and downregulation of vimentin in cervical cancer cell lysates exposed to varying concentrations (500, 750, 1000 µM) of naringin. Since β-catenin influences the cell cycle regulation, we next wanted to

observe the effect of naringin treatment on cell cycle. Our results demonstrate a drastic reduction in CDK4, cyclin D1, c-Myc, and CDK2 expression and subsequently increases the expression of p21/cip1 and p27/Kip1 proteins which acts as gatekeepers of the cell cycle (Fig. 4B). Further to support the above results, cell cycle analyses were performed. After cell synchronization in low serum-containing medium, CC cells were allowed to progress through various cell cycle phases in serum-containing medium for at least 3 h. The cells were then treated with varying concentrations (500, 750, 1000 µM) of naringin for 24 h. Our results demonstrated that naringin arrests $G_0/G_1$ phase of the cell cycle in CC cells (Fig. 4C and 4D) and the quantification of cells in various cell cycle phases showed a significant population of cells was arrested at $G_0/G_1$ phase of the cell cycle.
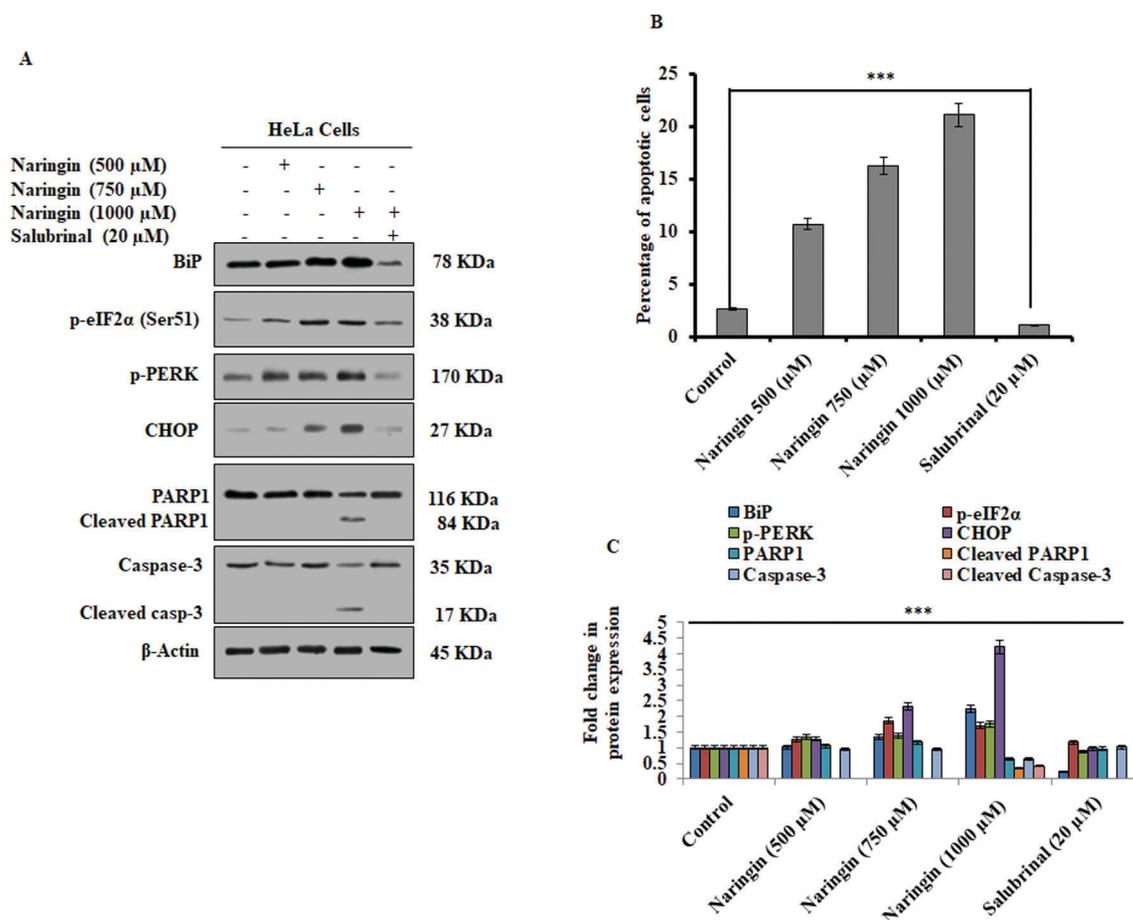
**Figure 3. Naringin triggers ER stress in CC cells**
(**A**) Immunoblotting analysis of CC cells (HeLa) exposed to various dose-dependent concentrations of naringin shows the steady increase in expression of BiP, CHOP, ATF-4 and phosphorylation of p-eIF2α, p-PERK proteins along β-actin as an internal control. (**B**) Bar diagram presents the quantification of the apoptotic cell population after exposure to varying concentrations of naringin in different settings. (**C**) Densitometry analysis represented by histogram shows the fold change in protein expression after normalized with β-actin. The data denotes the mean value ±S.E. of 3 independent unbiased experiments. ***$p<0.001$

## DISCUSSION

Despite significant advancement have been attained in the therapeutics of cancer such as surgery (Hussain *et al.,* 2014), radiotherapy (Baskar *et al.,* 2012), chemotherapy (Gandhi *et al.,* 2014), targeted therapy (Kim *et al.,* 2015), and recently immunotherapy (Parish *et al.,* 2003); however, the conventional approach has awful deleterious effects and develops drug resistance (Holohan *et al.,* 2013). Therefore, the main purpose of the advancement in cancer therapeutics is the discovery of novel approaches or drugs that kills cancer cells specifically. Owing to less adverse effects on normal cells, and evade drug resistance, in recent times focus has been shifted towards natural products to use them as anticancer agents (Mehta *et al.,* 2010; Ozben *et al.,* 2006). Although numerous agents from the natural source have been tested for anticancer potential most of the natural compounds, their underlying mechanism of cell death is yet to be elucidated. Here, in the current study, we evaluated the antiproliferative potential of naringin in cervical cancer. Despite recent evidence showed the antiproliferative potential of naringin exhibited in a wide range of tumor cells, however, the current study evaluated the molecular mechanism associated with the cell death promoted by naringin in CC cells.

The current study revealed that naringin not only exhibits antiproliferative potential against CC cells by decreasing cell viability significantly but also exhibits safe toxicity in normal CC cells. Moreover, naringin induces ER stress-associated apoptosis in CC cells by increasing the expression and phosphorylation of key ER stress sensors (BiP, p-PERK, p-eIF2α) which eventually activates downstream target CHOP a crucial executioner of ER-dependent apoptosis. Additionally, naringin abrogates the β-catenin signaling pathway by inhibits phosphorylation of β-catenin at Ser675 and GSK3-β at Ser9, thereby triggers the arrest of the cell cycle at the $G_{0/}G_1$ phase of CC cells.

First, we studied the influence of naringin on cellular proliferation of CC cells. The higher concentration of naringin significantly decreases the cell viability of CC cells. However, in normal cervical cells, naringin decreases 50% cell proliferation at very high concentrations (above 5000 μM), indicates that naringin showed a safe toxicity profile. Various studies have revealed that natural compounds obtained from plant sources exhibit antiproliferative effects by inducing UPR associated with ER stress-dependent apoptosis in cancer cells (Farooqi *et al.,* 2015; Rasool *et al.,* 2017). Mechanistically, UPR associated ER stress produced by natural compounds disrupts the association of ER-resident chaperone protein BiP and ER sensors PERK, ATF6 and IRE1 inside
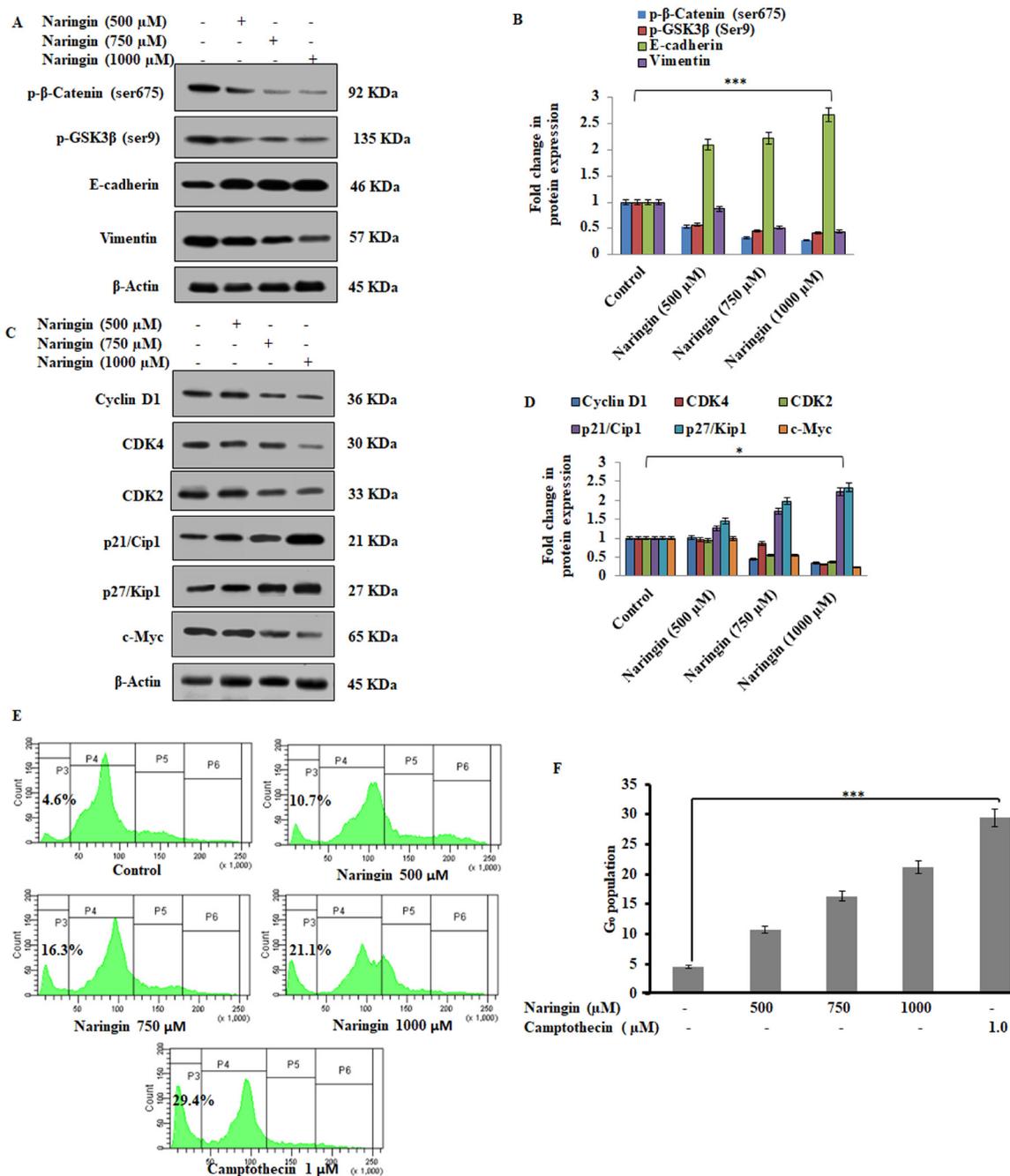
**Figure 4. Naringin inhibits β-catenin signaling pathways and cell cycle arrest in CC cells**
(**A**) Immunoblotting analysis of CC cells (HeLa) exposed to varying concentrations of naringin, shows the expression pattern of β-catenin and associated proteins (E-cadherin, Vimentin, and GSK3-β) along β-actin as an internal control. (**B**) Densitometry analysis represented by histogram shows the fold change in protein expression after normalized with β-actin. (**C**) Immunoblotting analysis of CC cells (HeLa) exposed to various concentrations of naringin, shows the expression profile of cell cycle regulation proteins (CDK4, cyclin D1, c-Myc, CDK2, p21/Cip1, and p27/Kip1) along β-actin as an internal control. (**D**) Densitometry analysis represented by histogram shows the fold change in protein expression after normalized with β-actin. (**E**) CC cells (HeLa) were treated with varying concentrations of naringin and subjected to cell cycle analysis for 24 h along with camptothecin as a positive control. (**F**) The bar diagram presents the quantification of CC cells in the G0 phase of the cell cycle after exposing them to various naringin concentrations. The data denotes the mean value ±SE of 3 independent unbiased experiments. *$p<0.05$, ***$p<0.001$

the lumen of ER (Chevet *et al.,* 2015; Shore *et al.,* 2011). These sensor proteins modulate ER stress as per the demand. If the stress is mild cells try to restore protein folding by activating the ER axis which increases the expression of chaperone proteins and restores ER stress in favor of survival, however, if the stress is severe, the cells activate ER axis which is associated with CHOP and induces CHOP mediated apoptosis in these cells

(Tabas *et al.,* 2011). Consistent with recent data, the current study demonstrates that naringin induces ER stress-mediated apoptosis. Subsequent investigation revealed that the naringin increases the expression of chaperone protein BiP and CHOP and augments phosphorylation of PERK and eIF2α. Further, investigation demonstrates that the cleaved product of caspase-3 was prominent in cell lysate treated with a higher concentration of nar-

ingin, which was earlier probed with ER stress markers, indicates that execution of apoptosis is dependent on ER stress. Additionally, we observe pretreatment with ER stress inhibitor salubrinal and followed by naringin treatment decreases the apoptotic cell population significantly. This strengthens our hypothesis that naringin induces ER stress-mediated apoptosis.

Deregulation of signaling pathways due to mutations causes the upregulation of many oncoproteins in cancer cells (Kikuchi *et al.*, 2008; Nayak *et al.*, 2017). Among them, Wnt/β-catenin signaling upregulation is very crucial in many cancers including cervical cancer. The upregulation of β-catenin enhances tumor cell progression, survival, invasion, and metastasis in cervical cancer (Gavert *et al.*, 2008; Liu *et al.*, 2010). Many studies have revealed that β-catenin expression increases during the cell cycle progression and influences cell cycle regulators to increase DNA content for cellular division and growth of cells (Kimura *et al.*, 2006). Another finding revealed that withanolide D arrests cell cycle in pancreatic cancer cells (Sarkar *et al.*, 2014), further results showed that the arrest of the cell cycle was due to attenuation of the β-catenin pathway. Consistent with these findings, our results demonstrate that a higher concentration of naringin abrogates phosphorylation of β-catenin at ser675 and GSK3-β at Ser9 thereby attenuates β-catenin signaling. Further, the study reveals that naringin treatment upregulates E-cadherin and downregulates Vimentin expression in CC cells. Additionally, naringin treated cells at higher concentrations reduce the expression of prosurvival proteins c-Myc, CDK2, CDK4, and cyclin D1 which are involved in cell cycle and concomitantly increases the cell cycle checkpoint regulator proteins p21 and p27 which eventually curtails cell cycle to progress and arrests at $G_0/G_1$ phase in CC cells.

In conclusion, the current study revealed that naringin displayed a significant antineoplastic effect in CC cells and are less toxic to normal transformed cervical cells. Mechanistically, naringin promotes ER stress-associated apoptosis to enhance cell killing in CC cells. Additionally, naringin attenuates the β-catenin pathway by dephosphorylating β-catenin at Ser675 and GSK3β at Ser9 respectively. Further, naringin augments protein expression of cell cycle checkpoint regulators (p21/Cip, p27/Kip) and curtails cell cycle at the $G_0/G_1$ phase in CC cells. Thus, our results provide a piece of strong evidence for further investigation of the use of naringin as a potential chemotherapeutic agent for human cervical cancer.

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## Authors Contributions

Ruyin Lin: Conceptualization, Visualization Data curation, acquisition, Formal analysis. Xinxin Hu: Conceptualization, Writing, review & editing. Shaorong Chen: Conceptualization, design experiments, writing review and editing the original draft. Qiyang Shi and Huiqing Che formal analysis helps in experiments and reviewing the manuscript.

## Conflict of interest

The authors declare no conflict of interest.

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