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Original Research Article

Antiproliferative and Apoptotic Effects of Angelicin in Highly Invasive Prostate Cancer Cells

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Abstract

Purpose: To demonstrate the anti-proliferative activity of angelicin against human prostate cancer (PC-3) cells and to evaluate its mechanism
Methods: MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) assay was used to assess the anticancer activity (growth inhibition) of angelicin in PC-3 cells. Fluorescence microscopy using Hoechst 33258 staining and inverted phase microscopy was employed to evaluate the effect of angelicin on nuclear morphology. Flow cytometry, using propidium iodide, was employed to study cell cycle-related effects of angelicin. Apoptosis induction by angelicin was examined by annexin V/PI assay.
Results: Angelicin induced potent growth inhibitory effects in human prostate cancer (PC-3) cells in a dose-dependent manner. Angelicin-treated cells exhibited chromatin condensation which implied an early apoptotic event. Inverted phase microscopy revealed that reduction of cell population occurred with increase in the angelicin dose. Flow cytometry results showed that angelicin induced cell cycle arrest in the sub-G1 phase. Angelicin induced both early and late apoptosis in PC-3 cells following a dose-dependent pattern.
Conclusion: Angelicin inhibits the growth of PC-3 human prostate cancer cells in vitro by inducing early and late apoptosis, cell cycle arrest and chromatin condensation.

Keywords: Angelicin, Coumarin, Prostate cancer, Anticancer activity, Apoptosis, Cell cycle arrest, Chromatin condensation, Flow cytometry

INTRODUCTION

Prostate cancer is the development of cancer in the prostate, a gland in the male reproductive system. Most prostate cancers are slow-growing; however, some grow relatively fast. The cancer cells may spread from the prostate to other parts of the body, particularly the bones and lymph nodes. In the advanced stages of this cancer, the symptoms may include blood in the urine, pain in the pelvis, back or when urinating and difficulty in urinating [1,2]. Prostate cancer (PC) is the principal cause of new cancer cases and the second most common cause of cancer-related deaths in men in USA [3]. Prevalence rate for clinical prostate cancer in Western men are almost 40 times higher than those for Asian men. The regular treatment opportunities for prostate

cancer comprise of surgery and radiotherapy, but these treatment regimens are loaded with severe side-effects. Plant-based drugs may provide better treatment options by preventing or slowing down disease progression. About 40 - 45 % of patients suffering from prostate cancer use traditional complementary and alternative medicine (usually plant based) as an additional therapy [4,5].

Since limited treatment regimens are available for highly invasive prostate cancer and loaded with deleterious side-effects, however the complementary and alternative medicine may provide better treatment options by inhibiting or slowing down disease progression. According to a recent survey, around 30 – 40 % of patients suffering from prostate cancer use complementary and alternative medicine as an additional therapy our objective was to focus on a plant-based coumarin, angelicin which is actually a furanocoumarin we evaluated the antiproliferative effect of angelicin against highly invasive human prostate cancer (PC3) cells. The effect of furanocoumarin on apoptosis, cell cycle phase distribution and cell migration was studied. Angelicin showed a potent growth inhibitory effect against these cancer cells (Fig. 1). The number of living healthy cells decreased dramatically with the increase in the concentration of angelicin with an IC_{50} value of 65.2 µM after the cells were exposed to angelicin for 48 h.

EXPERIMENTAL

Drugs and reagents

Angelicin was purchased from Sigma Aldrich (St. Louis, MO, USA). It was dissolved to a concentration of 50 mM in 100 % dimethyl sulfoxide (DMSO) as a stock solution and stored at 20 ˚C. The final DMSO concentrations used in the present study were ≤ 0.1 %.

Cell line, culture conditions and angelicin treatment

Human prostate cancer (PC-3) cell line was purchased from Guangdong Medical College (Zhanjiang, China). The cells were cultured in RPMI 1640 (Gibco BRL, Grand Island, NY, USA), supplemented with 10 % (v/v) fetal bovine serum (Gibco BRL), penicillin 100 U/mL and streptomycin 100 U/mL and maintained in a humidified atmosphere of 95 % air and 5 % CO_2 at 37 ˚C. When the proliferation of the cells was 70-80 %, the cells were treated with various

concentrations of angelicin (5, 25, 50, 75 and 100 µM) for 48 h.

MTT assay for cell viability

PC-3 cell density was adjusted to 2×10^5 cells per 100 µL. Cells were seeded onto 96 well plates, which were placed in an incubator overnight to allow for attachment. The cells were pretreated with 5, 25, 50, 75 and 100 µM angelicin for 48 h and MTT was then dissolved to a concentration of 5 mg/mL in assay medium. A total of 20 µL MTT solution was transferred to each well to yield a final volume of 120 µl/well. Plates were incubated for 4 h at 37 ^{o}C in 5 % CO_2. Following incubation, supernatants were removed and 150 µL DMSO was added. Plates were then placed on an orbital shaker for 10 min and the absorbance was recorded using the ELISA plate reader (PerkinElmer, Inc, Waltham, MA, USA) at 595 nm.

Evaluation of angelicin-induced cell apoptosis

Human prostate cancer cells which were grown on coverslips in 12-well plates were exposed to different concentrations (0, 25, 50 and 100) of angelicin for 48 h, then incubated with Hoechst 33258 (Hoechst Staining Kit, Beyotime, China). Fluorescence microscopy was employed to detect and measure cell shape captured from different random visual fields. The ratio of apoptotic cells to total cell number was calculated.

Inverted light microscopy

PC-3 cells were seeded in 6-well plates at 2×10^4 cells per well in 5 mL of complete growth medium, incubated for 24 h and treated with angelicin at various concentrations (0, 25, 50 and 100 µM). Control cells treated with 0.1 % DMSO alone were also included. The morphological changes were observed under an inverted light microscope (Olympus, Center Valley, PA, USA) after 48 h.

Determination of the effect of angelicin on cell cycle arrest in PC-3 cells

PC-3 cells (1×10^6 cells/mL) were seeded into each well of 6-well plates and incubated for 24 h for cell attachment and recovery. The cells were treated with different concentrations (0, 25, 50 and 100 µM) of angelicin. Untreated cells (control) were also incorporated. After incubation for 24 h, the cells were harvested and fixed with ice-cold 70 % ethanol (2 mL) at −20 °C for 1.3 h Prior to analysis, the cells were washed with cold

PBS and re-suspended in 400 µL of PBS, 50 µL PI and 50 µL RNase A. The DNA contents were recorded by a flow cytometer (Becton Dickinson) equipped with Cell Quest software.

Annexin V-propidium iodide assay

Annexin V-FITC Apoptosis Detection Kit (BD Biosciences) was utilized for evaluating the mode of cell death induced by angelicin (apoptotic or necrotic cell death). The PC-3 cells (1×10^6 cells/mL) were seeded into each well of 6-well plates and incubated for 24 h at 37 $^{\circ}$C. The cells were treated with different concentrations (0, 25, 50 and 100 µM) of angelicin. The untreated and treated cells were harvested and washed with cold PBS. Subsequently, the cells were mixed with 180 µL of pre-diluted binding buffer containing Annexin V-FITC (10 µL) and of PI (10 µL) and further incubated for 15 min at 37 $^{\circ}$C in the dark. Subsequently, 200 µL of binding buffer was added into each tube. The percentage of cell undergoing apoptosis and necrosis was quantified using a flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA) equipped with Cell Quest software within 2 h.

Statistical analysis

Data are presented as mean ± standard deviation (SD, n = 6). Analysis of variance (ANOVA) was conducted using SPSS (version 20 SPSS Inc, Chicago, DE USA) to evaluate significant difference. Subsequently, the significance of the difference was evaluated using Dunnet test, and $p < 0.05$ was considered significant.

RESULTS

Antiproliferative activity of angelicin against human prostate cancer (PC-3) cells

Angelicin showed a potent growth inhibitory effect against these cancer cells (Fig. 1). The number of living healthy cells decreased dramatically with the increase in the concentration of angelicin with an IC_{50} value of 65.2 µM after the cells were exposed to angelicin for 48 h.

Detection of angelicin-induced apoptosis by fluorescence microscopy

Angelicin-treated cells exhibited chromatin condensation or dense staining fragmentation (apoptotic bodies), which implied an early apoptotic event. The induced apoptosis seemed to follow concentration dependence with greater apoptosis at higher angelicin doses (Fig. 2).
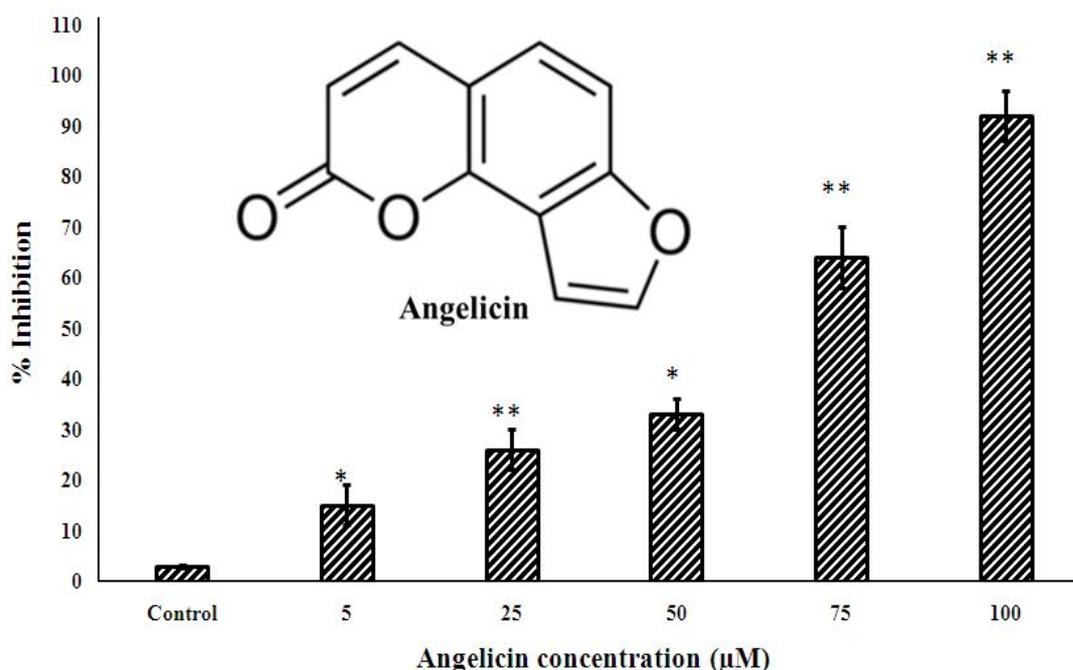

Figure 1: Effect of angelicin on the cell viability of human prostate cancer cell (PC-3) at different concentration; *p < 0.05 vs. control group; **p < 0.01 vs. control group
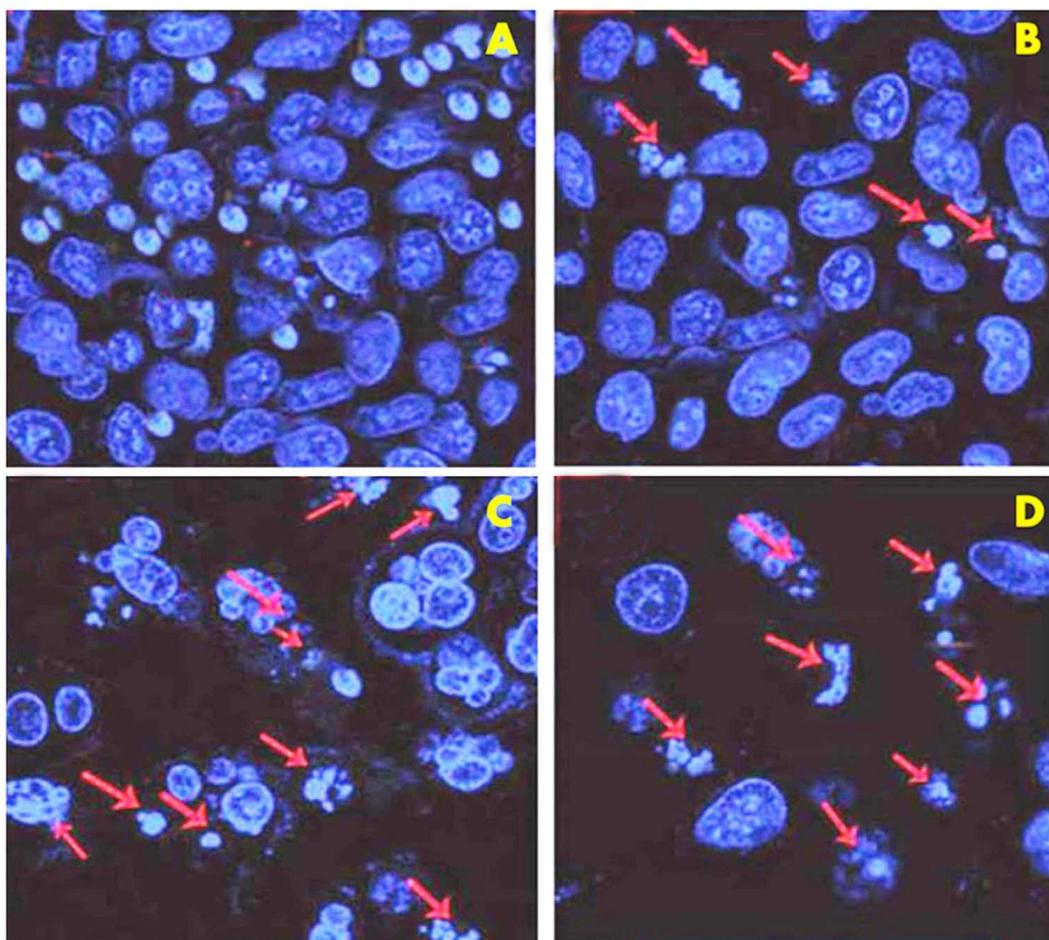
Figure 2: Morphological observation of angelicin-induced apoptosis with Hoechst 33258 staining at actual magnification 200×. PC-3 cells were treated without (A) and with angelicin 25 μM (B), 50 μM (C), and 100 μM (D) for 48 h. Red arrows represent chromatic condensation

Inverted light micrographs

The most prominent changes characteristic of apoptosis were observed in the treated cells that include the detachment of the cells from substratum, cell shrinkage, nuclear condensation, membrane blebbing as well as formation of apoptotic bodies. As revealed by inverted light microscopy, the untreated control cells were evenly distributed on the substratum. Reduction in the cell population was noted with increase in the angelicin concentration. Angelicin induced growth inhibitory effects and morphological alterations in human prostate (PC-3) cancer cells (Fig 3A - D), cell shrinkage and blebbing was increased with increase in angelicin concentration.

Angelicin induces cell cycle arrest in PC-3 cells

It was observed that angelicin induced cell cycle arrest in the sub-G1 phase of the cell cycle increasing cell population of the sub-G1 phase after 48 h (Fig. 4). In the control group, the major cell population was found to be in the G1 phase (67.12 %), with a low percentage of cells in the G2/M phase. Generally, the cell cycle arrest is associated closely with apoptosis, that is, the occurrence of cell cycle arrest leads to cell apoptosis, which includes numerous signaling molecules and regulatory proteins.

Apoptosis results

The results in Fig. 5 reveal that angelicin induced both early and late apoptosis in a concentration-dependent manner (Fig 5B - D) as compared to the untreated control cells (Fig 5A). The percentage of apoptotic cells increased from 13 % in the untreated control to 25.7, 64.5 and 78.3 % in 25, 50 and 100 μM angelicin concentration-treated cells, respectively.
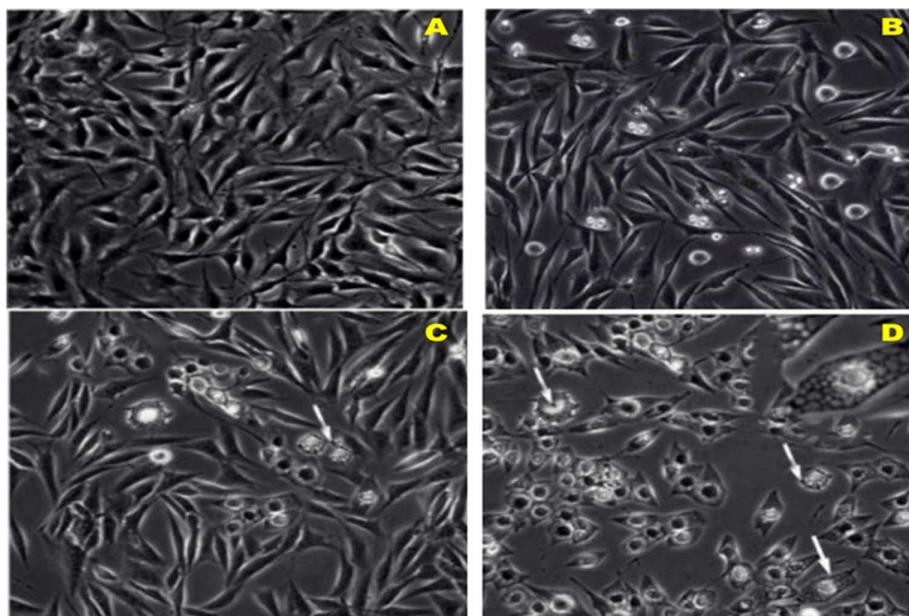
Figure 3: Angelicin-induced morphological changes in human prostate cancer (PC-3) cells as detected by inverted light microscopy (magnification X400). Cellular shrinkage and blebbing were observed in angelicin-treated cells (arrows). A represents control (untreated cells), B,C and D represent effect of 25, 50 and 100 µM of angelicin on cell morphology of PC-3 cells
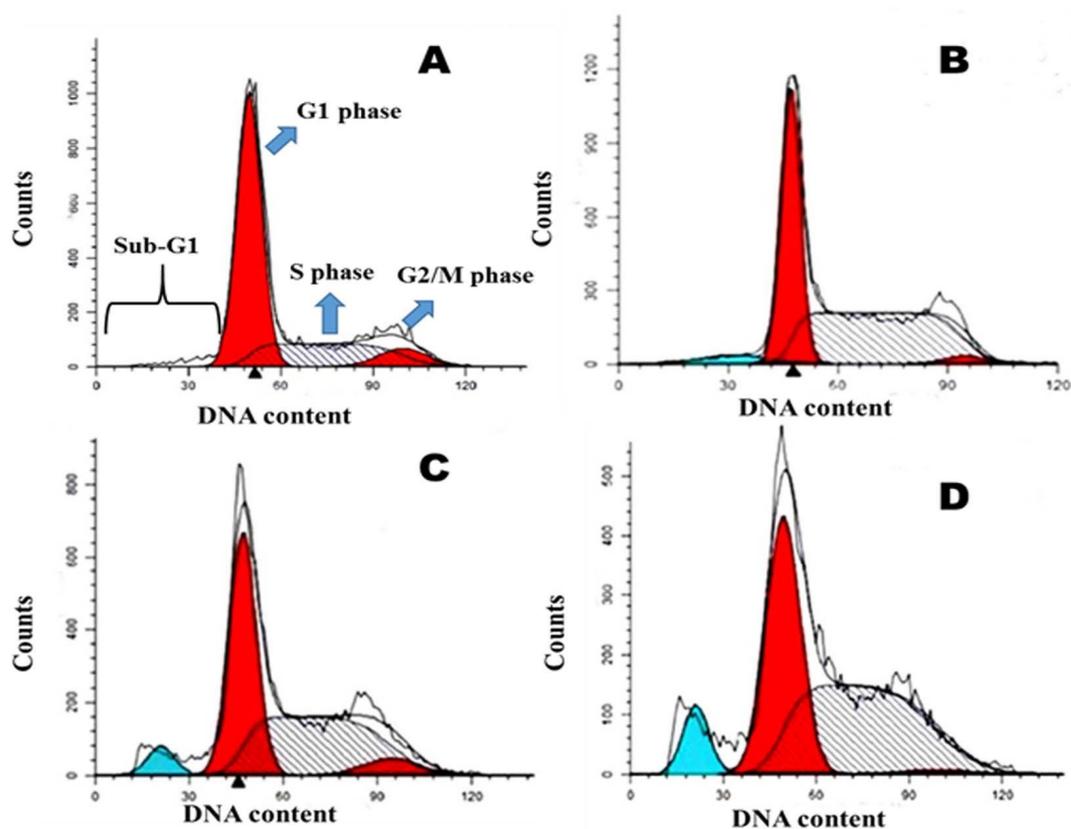

Figure 4: Angelicin induced cell cycle arrest at sub-G1 phase in human prostate cancer cells (PC-3). The PC-3 cells were subjected to 0 µM (untreated, A), 25 µM (B), 50 Mm (C) and 100 µM (D) of angelicin. The cells in the sub-G1 phase (apoptotic cells) increased from A-D, with an increase in angelicin dose. The DNA histogram shows the distribution and the percentage of cells in phases of the cell cycle
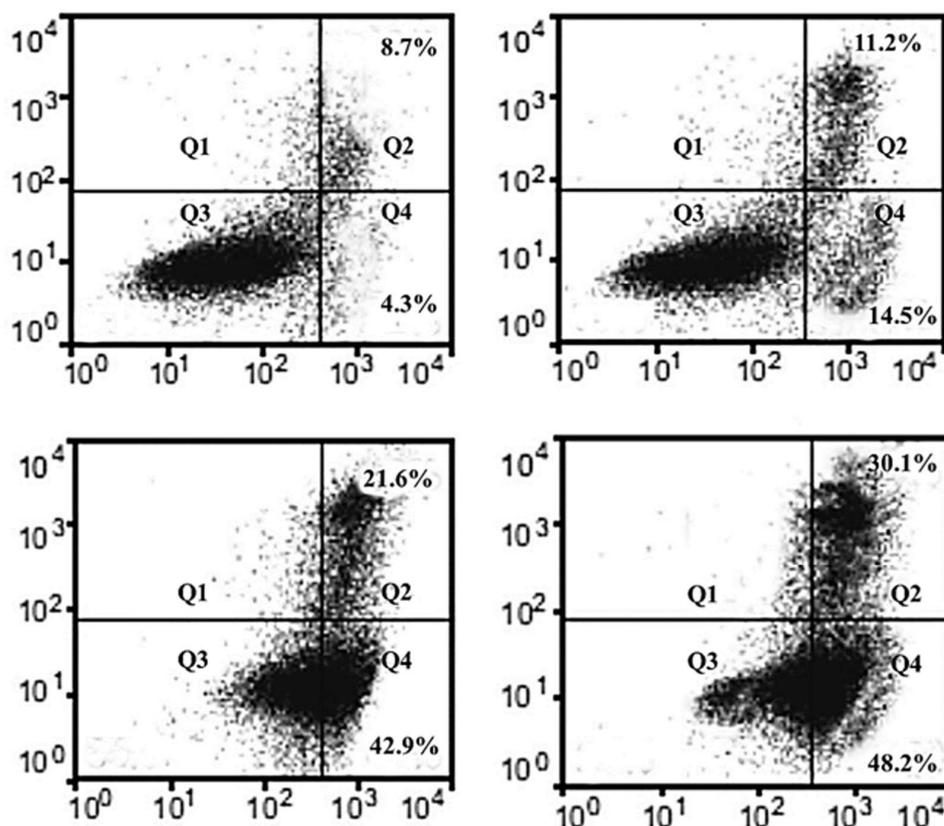
Fig 5: Apoptosis induction in human prostate (PC-3) cancer cells by angelicin. PC-3 cells were exposed to different doses of angelicin (0, 25, 50 and 100 µM) for 48 h and analyzed by flow cytometry with annexin V-FITC/PI staining. The QI quadrant shows necrotic cells, Q2 shows late apoptotic cells, Q3 shows live cells while as Q4 shows early apoptotic cell population. The plots in the figure are representative of 5 independent experiments

DISCUSSION

Previous studies have shown that some natural chemopreventive agents can induce apoptosis of tumor cells and inhibit tumor growth, both *in vitro* and *in vivo* [6-9]. Because of their selectivity in killing tumor cells and minimal toxicity compared to conventional chemotherapies, they are becoming promising approaches for tumor treatments. Coumarins and their known metabolite, 7-hydroxy-coumarin, have been shown to have growth suppressive effect on many cancer cell lines, such as colon-carcinoma cell lines, hepatocellular carcinoma cell lines, leukemia cell lines, melanoma cell lines, renal cell carcinoma cell lines and non-small cell lung carcinoma cell lines. Auraptene, one of the coumarins, has been shown to be effective in inhibiting the development of esophageal tumors and colitis-related colon cancers in animal models [10-12]. Coumarin has also been used in a clinical trial to prevent disease recurrence in melanoma patients [13]. They can affect multiple signaling pathways, such as ERK/MAPK and PI3K/Akt pathways, which play important roles in carcinogenesis [14-16].

In the present study, anticancer effect of angelicin-a known coumarin, was evaluated against human prostate cancer cells (PC-3) using MTT assay. Further, the underlying mechanism of the anticancer action of angelicin was demonstrated by studying the effect of this coumarin on nuclear morphology of the PC-3 cells after drug treatment. For this purpose, fluorescence and inverted phase microscopy were used. The results revealed that angelicin-treated cells exhibited chromatin condensation which implied an early apoptotic event. Inverted phase microscopy revealed that reduction of cell population occurred with increase in the angelicin dose. Further flow cytometry experiment revealed that angelicin induced cell cycle arrest in the sub-G1 phase of the cell cycle. Generally, the cell cycle arrest is associated closely with apoptosis, that is, the occurrence of cell cycle arrest leads to cell apoptosis, which includes numerous signaling molecules and regulatory proteins.

CONCLUSION

The results of this study demonstrate that angelicin, a naturally occurring coumarin, exhibits anticancer effects against PC-3 human prostate cancer cells by inducing apoptosis, chromatin condensation and cell cycle arrest. Further studies are required to develop this potent compound as a possible chemotherapeutic agent for treating prostate cancer.

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