

ORIGINAL ARTICLE

Anticarcinogenic effect of Umbelliferone in human prostate carcinoma: An *in vitro* study

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Summary

Purpose: To explore the chemoprotective effect of umbelliferone (UF) on prostate cancer cell lines, i.e. primary stage (LnCap) and last stage (PC3) prostate cancer together with the effect on the induction of apoptosis and alteration on cell cycle arrest.

Methods: Various concentrations of UF were evaluated against the different prostate cancer cell lines. Lipopolysaccharide (LPS) induced cytokines related factor profiling, proinflammatory cytokines, and inflammatory mediators were studied using Western blot analysis.

Results: UF showed significant apoptotic effect. Moreover, treatment with UF did not show apoptosis or cell cycle ar-

rest on the non-cancerous cells including BHP-1, suggesting a selective tumor cell specific effect. UF treatment also enhanced the expression of Bax in PC3 cells, but had no significant effect on the activation of nuclear factor κ B (NF- κ B). Thus, the apoptosis induction was independent of NF- κ B activation.

Conclusion: The results of the present investigation confirmed the chemoprotective effect of UF in early-stage (LnCap) and late-stage (PC3) prostate cancer cells.

Key words: apoptosis, nuclear factor kappa B, prostate cancer, umbelliferone

Introduction

Prostate cancer (PCA) is a very common malignancy, and is considered as the leading cause of cancer-related deaths across the globe. Due to limitations of available treatments each year half of the PCA patients ultimately progress to advanced disease [1]. The paracrine and autocrine expansion factor receptor cross-talk and associated mitogenic signaling play a crucial role in the apoptosis and proliferation of the PCA cells [2,3]. PCA gradually becomes hormone-resistant [4]. New treatment options against PCA generally target the apoptosis induction to control the invasiveness as well as the proliferation of PCA in advanced stages [5]. In this regard, plants and plant-based compounds attracted much attention for the treatment of numerous types of cancers.

According to an estimate, nearly almost one-third of anticancer drugs derive from natural products. Thus, much of the research has been focused on the mechanistic analysis of these chemical compounds in the protection/chemoprevention of various types of cancer, including PCA.

Much evidence from *in vitro* and *in vivo* studies suggests a protective effect of umbelliferone (UF) against various types of malignancies [6-10]. Chemically, UF is classified as a 7-hydroxy coumarin derivative and is associated with numerous pharmacological activities, such as antioxidant, antidiabetic, antihyperlipidemic [9], antinociceptive, antiarthritic and antiinflammatory [7,10], together with an effect on the allergic airway inflammation and killing of laryngeal cancer cells *in vitro* [11].

Prompted by the above, the current investigation was intended to evaluate the effectiveness of UF against prostate cancer cells via estimating its effects on cell cycle arrest, apoptosis and activation of NF- κ B pathway. The effect of UF was determined on the BPH-1, LnCap and PC3 cell lines. The current findings might help provide a new basis for utilization of UF as a chemopreventive agent against PCA.

Methods

Cell lines

The benign prostate hyperplasia-1 (BPH-1 line), prostate cancer epithelial cells (PC3 line) and early-stage androgen-dependent prostate cancer cells (LNCaP line) were procured from the American Types Culture Collection (ATCC, Manassas, VA, USA).

Cell lines culture

BPH-1 and PC3 cells were cultured in DMEM supplemented with 5% fetal bovine serum (FBS). The LNCaP cells were cultured in RPMI 1640 medium (Invitrogen, Carlsbad, CA) together with 5% FBS. All types of cells were stored at 37°C in 95% air and 5% CO₂ incubator.

Cells' treatment

UF (99%; Sigma Aldrich, USA) was used for the treatment against PCA cells. Different concentrations (50, 100 and 200 μ mol/L) of UF were used for the evaluation of possible activity against PCA cells. The control treatment consisted of DMSO at a final concentration matching the dose of treated cells and less than 0.1% of the final volume.

Cell viability assay

MTT [(3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) and electron coupling reagent were used for the estimation of cell viability. Briefly, the cells (5000) were seeded in 96 well-plates and incubated overnight and then exposed to different concentrations of UF for 24 hrs. The solvent was used as positive control and all experiments were performed in triplicate. The change in cell viability was calculated by determining the optical density (OD) ratio between the treated and untreated cells.

Lipopolysaccharide (LPS) stimulated cytokines related factor profiling

12 well-plates were used to re-culture the dermal fibroblasts to fabricate confluent monolayers in DMEM. UF was added in the culture media together with addition of LPS for 24 hrs and the media were harvested for ELISA test.

ELISA assay

The effects of UF on the proinflammatory cytokines IL-8 and IL-6 were estimated using the ELISA assay on culture supernatants according to the manufacturer's instructions (RayBiotechnorcross, GA, USA). The standard curve was plotted with supplied standards to allow conversion of the OD at 450 and the experimental values were calculated as pg/ml. All the experiments were performed in triplicate.

Cell cycle estimation

For the estimation of the cell cycle, UF and floating cells were fixed in ethanol (70%) (10⁶ cells/ml). The cells were again suspended after incubation for 12 hrs at -20°C, and stained with the RNase and propidium iodide. The cell cycle evaluation was performed using flow cytometer (FASCSanto™ II, BD Biosciences, San Jose, USA).

Western blot analysis

The protein level was determined using the Bradford method [12]. The Western blot technique was used for the estimation of the concentration of Bcl-2, Bax and the proteins of NF- κ B pathway such as p-I κ B α , p50, I κ B α , p65 and IKK β in treated cells [13]. Cell cycle markers cMyc and p53 were also estimated. Primary and secondary antibodies were purchased from the Bio-Rad, Hercules, CA, USA. Secondary antibodies were conjugated with protein and horseradish peroxidase and detected using Western Lightning (NuPAGE Novex, Invitrogen, Carlsbad, CA, USA).

Apoptosis assay

ELISA kit (Roche Applied Science, Indianapolis, IN, USA) was used for the estimation of DNA fragmentation and cell death using the manufacturer's instructions. The cells (200 μ l) were lysed using the lysis buffer and then incubated for 30 min at room temperature. After incubation, the lysate was centrifuged for 10 min at 2000 rpm. The supernatant (20 μ l) was transferred into streptavidin-coated microplate which were covered with an adhesive cover and incubated again for 2 hrs using a shaker at room temperature. The solution of each well was completely removed and washed three times with incubation buffer. The ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphoric acid) (Sigma, Aldrich, USA) solution was added in each well and the plate was incubated again on a shaker. After shaking, the intensity of the colored solution was used for the estimation of absorbance.

Determination of proliferation

The antiproliferative effect of UF was estimated using the resazurin assay (Corning, Lowell, MA, USA) [14]. The cells were seeded in 96 well-plates at a density of 10 \times 10⁴ cells per well and then were harvested up

to 50-70% confluence and treated with UF (10-200 $\mu\text{g}/\text{ml}$) dissolved in prepared T-medium [14]. After that, 1 mg/ml of resazurin was added into each well and incubated for 48 hrs after which the fluorescence was estimated at 530 nm.

Results

Umbelliferone-induced caspase activation and cell cycle arrest

In the current investigation, we observed that UF caused induction of apoptosis and cell cycle

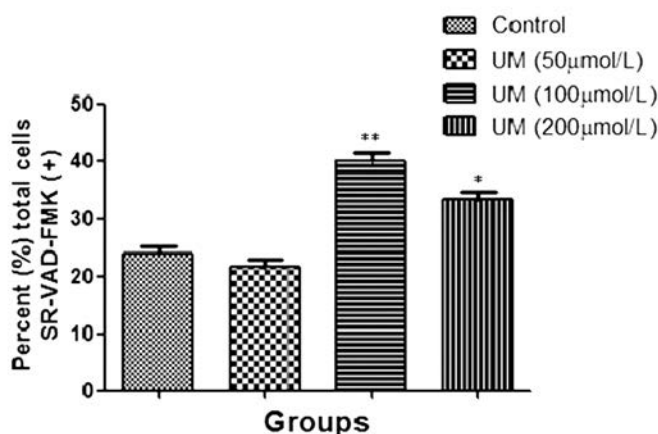


Figure 1. High doses of umbelliferone induced cell cycle arrest and apoptosis in benign prostate hyperplasia (BPH-1) cells treated with varying concentrations (50, 100 and 200 $\mu\text{mol}/\text{L}$) for 48 hrs, and labeled with SR-VAD-FMK stain (fluorochrome-conjugated caspase inhibitor) to assess multicaspase activity by flow cytometry. Values represent means \pm SEM, $n = 3$. * $p < 0.05$, ** $p < 0.01$ compared with control.

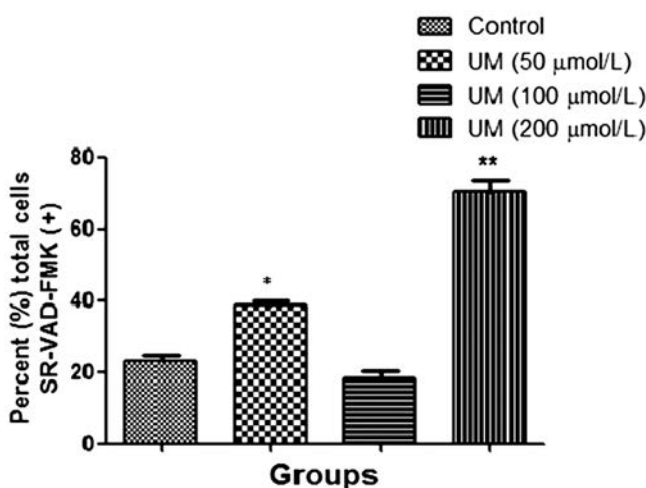


Figure 2. High doses of umbelliferone induced cell cycle arrest and apoptosis in LnCap cells treated with varying concentrations (50, 100 and 200 $\mu\text{mol}/\text{L}$) for 48 hrs, and labeled with SR-VAD-FMK stain (fluorochrome-conjugated caspase inhibitor) to assess multicaspase activity by flow cytometry. Values represent means \pm SEM, $n = 3$. * $p < 0.05$, ** $p < 0.01$ compared with control.

arrest. A UF dose of 100 $\mu\text{mol}/\text{L}$ was required to induce cell cycle arrest and caspase activation in PC3 and LnCap cell lines (Figures 1-3). On the other hand, UF (10 $\mu\text{mol}/\text{L}$) was used for the induction of the caspase activation in both cell lines. The PC3 (androgen-independent) cells treated with 10 $\mu\text{mol}/\text{L}$ of UF confirmed the activation of caspase and increase in the cell population in SubG1. Similarly, LnCap (androgen-dependent) cells indicated greater sensitivity to the caspase induction with UF. Interestingly, UF showed pro-apoptotic effects in both cell lines with more prominent pro-apoptotic effect in PC3 cells than in LnCap cells, identified based on the percentage of cells undergoing apoptosis. UF demonstrated increase of caspase activity in both PC3 and LnCap cells and exhibited apoptotic effect (Figures 4-6).

Antiinflammatory effect of umbelliferone

The anti-inflammatory effect of UF was determined on the dermal fibroblasts via estimating the capacity of LPS to induce the secretion of IL-8 and IL-6 cytokines. Control and control treated with UF did not show any changes in the level of cytokines. On the other hand, the LPS-treated group showed boosted level of cytokines, which was significantly ($p < 0.001$) reduced by UF (Figure 7).

Apoptosis effect of Umbelliferone

The apoptosis effect of UF was scrutinized on the prostate cancer cells. UF at different concentrations showed significant induction of apoptosis in both cell lines as compared to untreated cells (Figure 8).

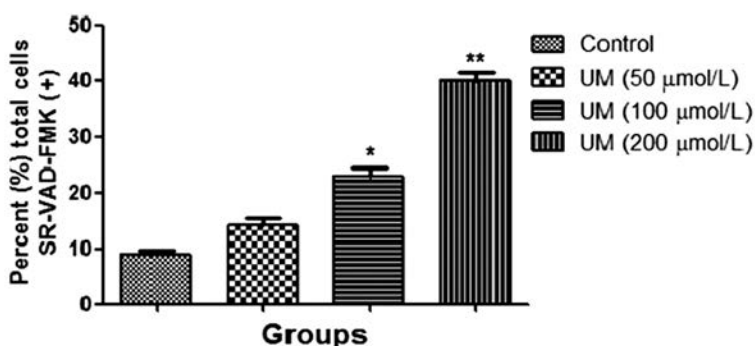


Figure 3. High doses of umbelliferone induced cell cycle arrest and apoptosis in PC-3 cells treated with varying concentrations (50, 100 and 200 $\mu\text{mol}/\text{L}$) for 48 hrs, and labeled with SR-VAD-FMK stain (fluorochrome-conjugated caspase inhibitor) to assess multicaspase activity by flow cytometry. Values represent means \pm SEM, $n = 3$. * $p < 0.05$, ** $p < 0.01$ compared with control.

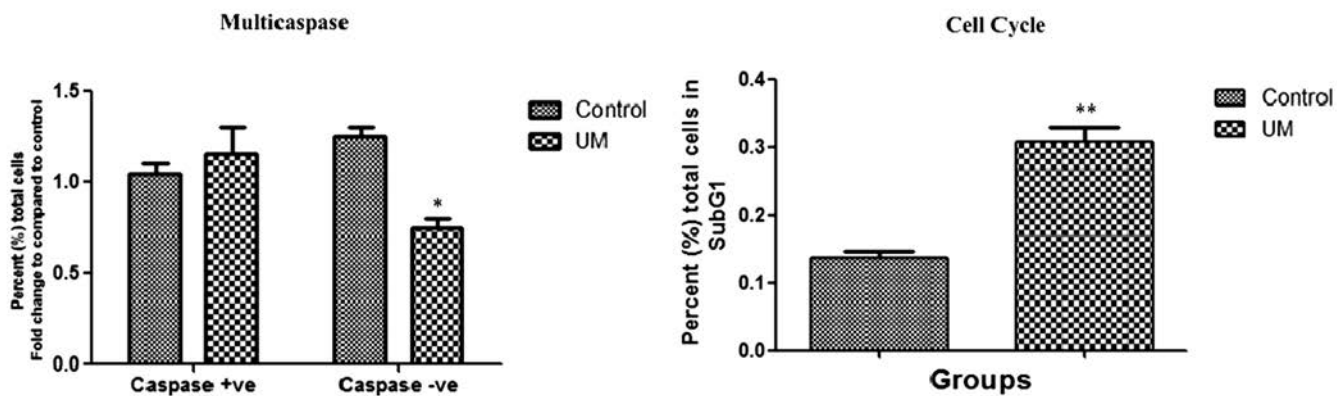


Figure 4. Umbelliferone induced apoptosis in BPH-1 cells. Multicaspase activity and cell cycle analysis were measured by flow cytometry. No significant changes in caspase activity or cell cycle were observed in BPH-1 cells treated with umbelliferone. Control treatment with 0.1% dimethyl sulfoxide. The data are presented as means±SEM, n = 3. *p<0.05, **p<0.01 compared with control.

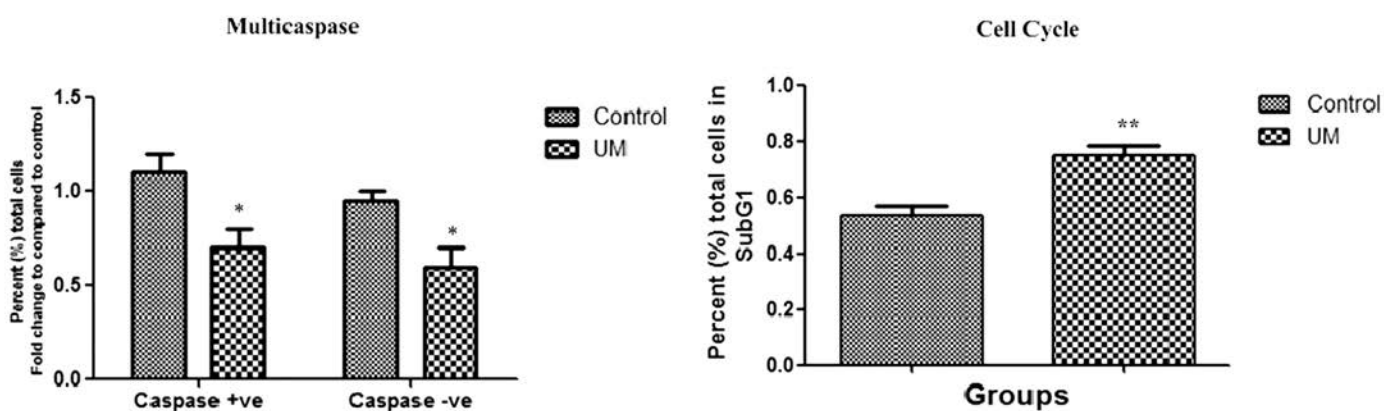


Figure 5. Umbelliferone induced apoptosis in LnCap cells. Multicaspase activity and cell cycle analysis were measured by flow cytometry. Control treatment with 0.1% dimethyl sulfoxide. The data are presented as means ± SEM, n = 3. *p<0.05, **p<0.01 compared with control.

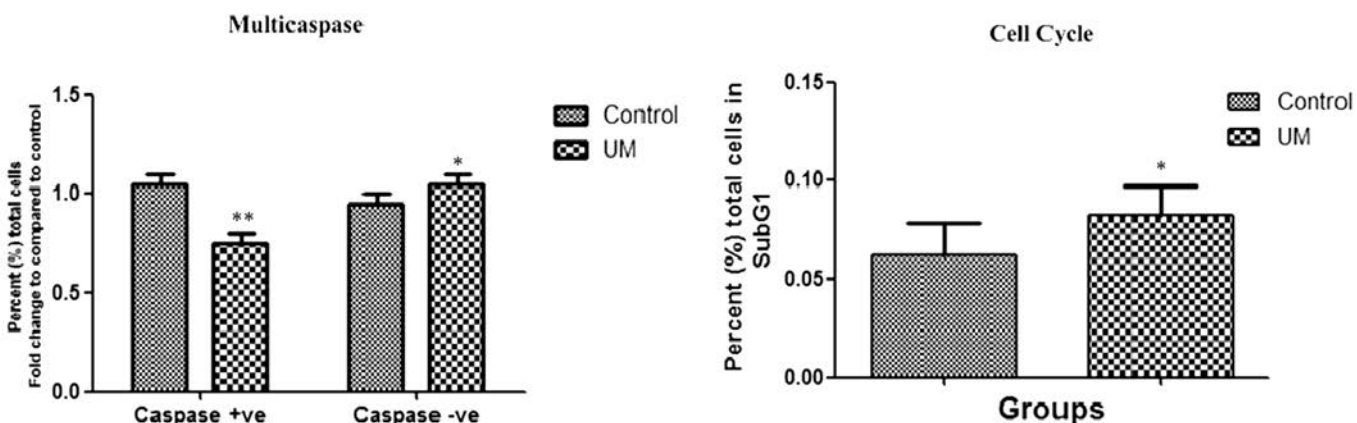


Figure 6. Umbelliferone induced apoptosis in PC3 cells. Multicaspase activity and cell cycle analysis were measured by flow cytometry. Control treatment with 0.1% dimethyl sulfoxide. The data are presented as means ± SEM, n = 3. *p<0.05, **p<0.01 compared with control.

Effect of umbelliferone on cell viability

MTT assay was used for the estimation of cell viability effect of UF on BPH1, LNCaP and PC3 cells. Figure 9 confirmed that UF did not show any cell viability effect on the BPH1 cells, whereas, on the other hand, LNCaP and PC3 cell treatment

with UF significantly decreased the cell viability in a dose-dependent manner.

Effect of umbelliferone on the BAX protein expression

To find out the possible mechanism of action of UF on the prostate cancer cells, we evaluated

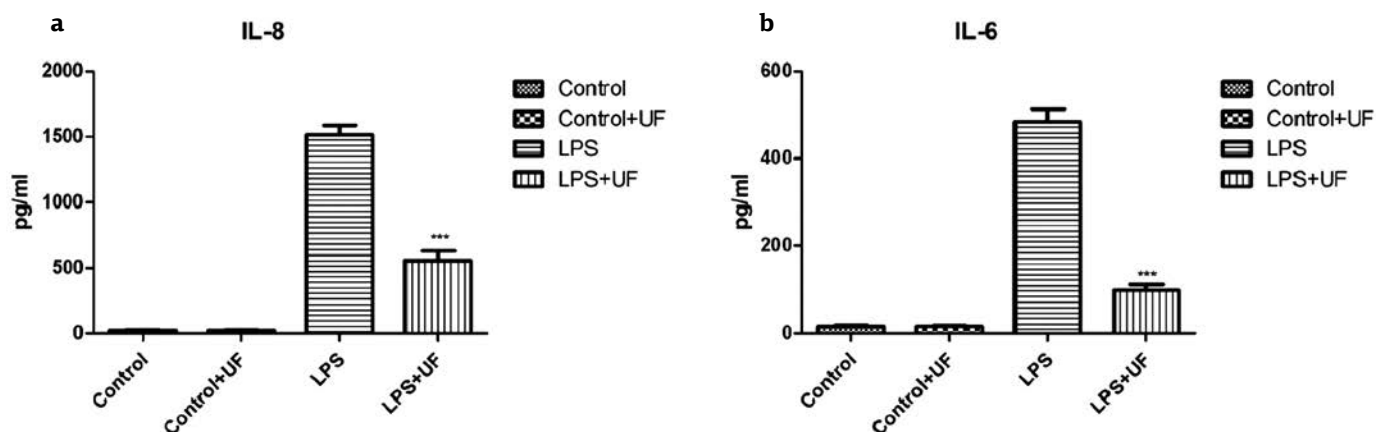


Figure 7. Determination of cytokines level in the dermal fibroblasts after 24 hr treatment with umbelliferone. Inhibition of lipopolysaccharides (LPS) stimulated deposition of sentinel cytokines by umbelliferone in dermal fibroblasts. Cytokines were determined in terms of altered level of (a) IL-8 and (b) IL-6 as described in Methods. *** $p < 0.001$ is considered extremely significant.

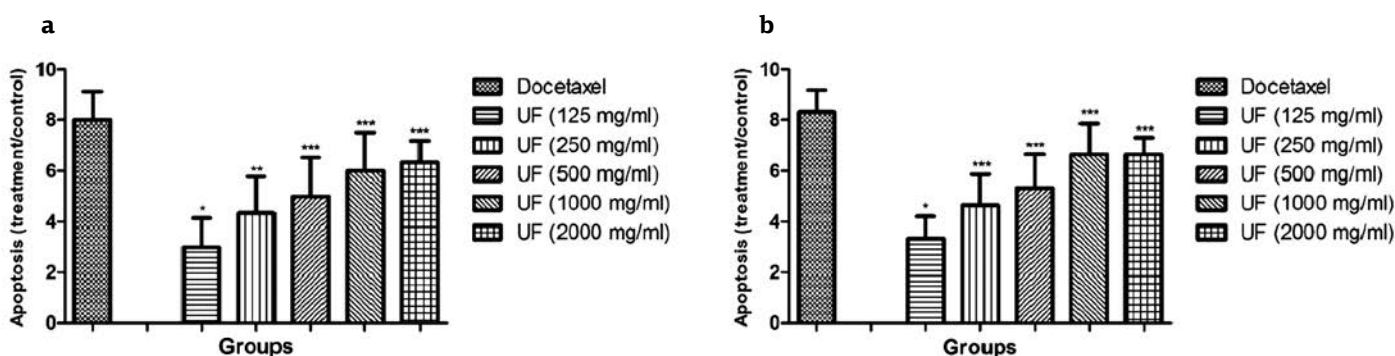


Figure 8. Apoptotic effects of umbelliferone in LNCaP and PC3 cells. LNCaP and PC3 cells were treated with different concentrations of UF (100–2000 $\mu\text{g/ml}$) for 48 hrs and ELISA was used for the estimation of cell death. Docetaxel was used as positive control. (a) LNCaP and (b) PC3 cells. Values represent means \pm SD. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ comparing with control (no treatment) group.

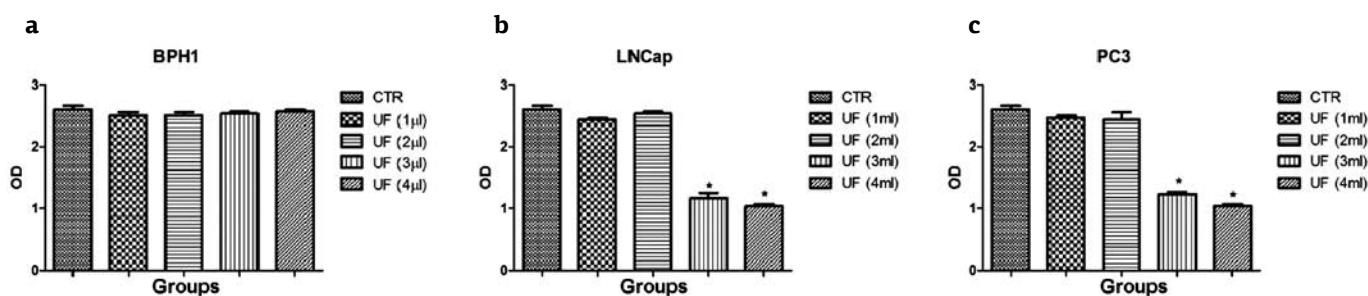


Figure 9. Umbelliferone showed cytotoxic effect against the LNCap and PC3 cells. The effects of umbelliferone on (a) BPH1, (b) LNCap and (c) PC3 cells were estimated as described in Methods. * $p < 0.001$ compared with control.

the protein expression of p53, cMyc, Bcl2 and Bax using the Western blot technique. The results showed that UF caused enhancement of BAX expression in PC3 cells while no effect was noticed in LNCap cells (Figure 10).

Effect of umbelliferone on the activation of NF- κ B pathways

The NF- κ B is used as the major mediator to evaluate the effect against apoptosis, cell prolifer-

ation and inflammation. The effect of UF on NF- κ B expression and the level of I κ B α , p-I κ B α and IKK β were also scrutinized. The results showed that UF significantly modulated the expression of NF- κ B as compared to control (Figure 11).

Discussion

In the current investigation we intended to compare the effects of different concentration of UF (50, 100 and 200 $\mu\text{mol/L}$) on prostate cancer

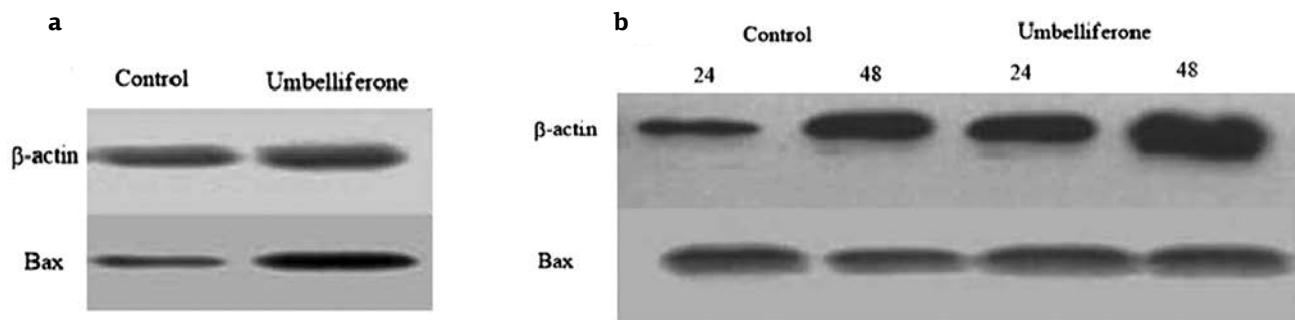


Figure 10. Umbelliferone augmented Bax expression. Western blot analysis was used for the estimation of protein expression levels of Bax nuclear extracts of treated cells. **(a)** LnCap and **(b)** PC3 cells were treated with 10μmol/L of umbelliferone. Control treatment consisted of media with <0.1% dimethyl sulfoxide. Umbelliferone increased Bax compared with control in PC3 cells, but not in LnCap cells. Representative images of triplicate experiments.

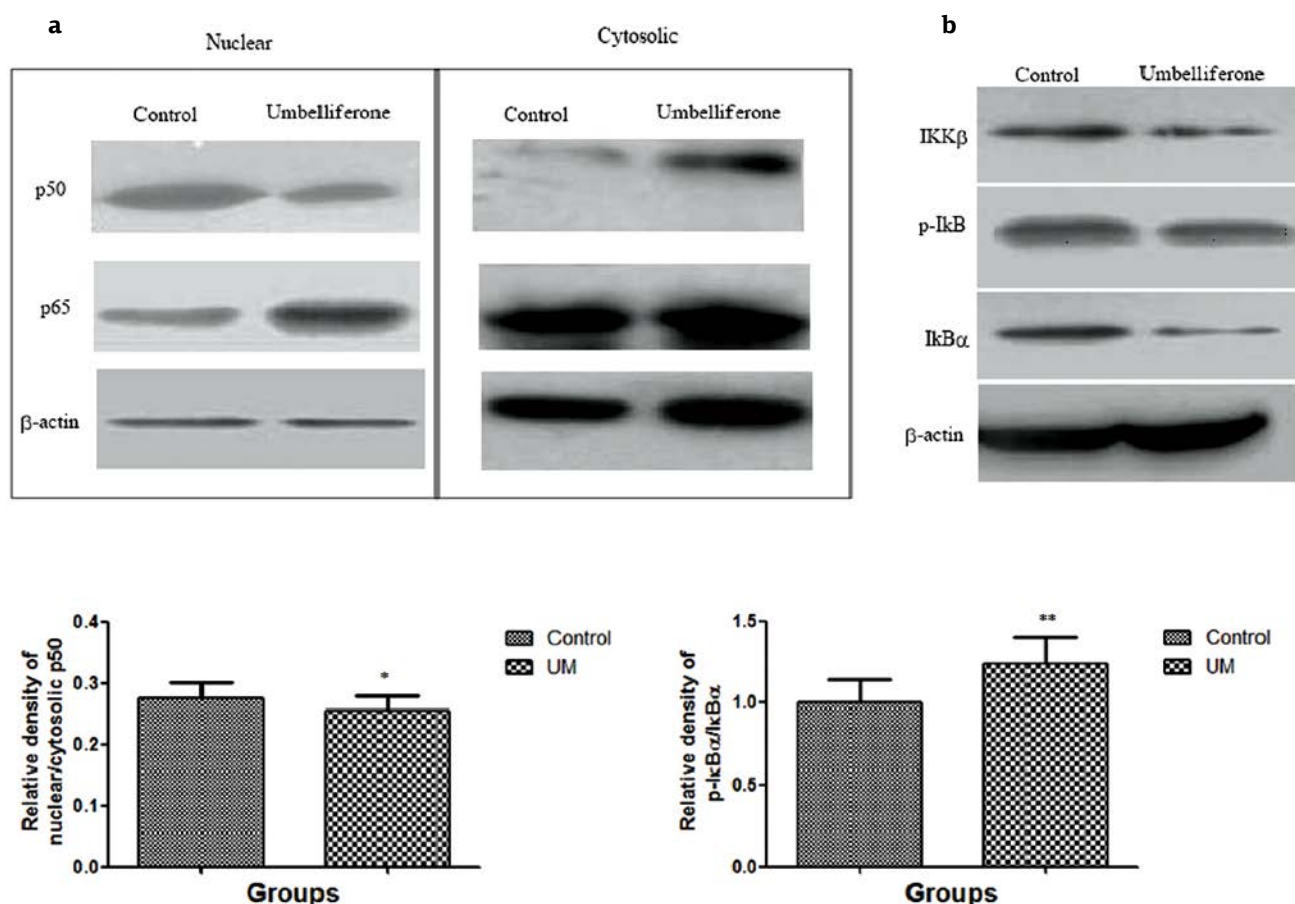


Figure 11. Umbelliferone didn't show any effect on NF-κB activation. **(a)** Western blot of nuclear and cytosolic extract from PC3 cells treated with dimethyl sulfoxide (control), 10μmol/L of umbelliferone against p65 and p50 antibodies. Relative density of nuclear/cytosolic p50 was obtained by dividing optical density of nuclear p50 to cytosolic p50 after normalized to respective β-actin. **(b)** Cytosolic extract of treated PC3 cells was raised against IKKβ, p-IκBα and IκBα. The relative density of p-IκBα/IκBα was obtained by dividing the optical density of p-IκBα to IκBα and normalized to β-actin. Daidzein increased nuclear p50 and cytosolic p-IκBα compared with control, but no significant changes were observed in cells treated with soy extract or genistein. Representative blots of triplicate experiments. Densitometry values represent means ± SEM, n = 3. *p<0.05, **p<0.01 compared with control.

cells. The results showed that UF was found to be more prominent in the induction of apoptosis in PC3 cells compared with the positive control. Additionally, UF treatment of the prostate cancer cell

lines showed antiproliferative effects, especially at late-stage prostate cancer cells (PC3) [10,15]. These results confirmed that UF may be more safe and effective in the prevention of PCA.

This study showed that UF induced caspase activation without modification in the Bax expression in LnCaP cells. On the other hand, UF could induce cell cycle arrest and caspase activation in PC3 cell lines via Bax and NF- κ B activation [16,17]. It also showed diverse effects on both cell lines (PC3 and LnCap cells) [18]. Moreover, UF demonstrated upregulation of Bax expression. A possible mechanism of UF activities may be correlated with the regulation of Bax expression and via targeting other downstream proteins, including inhibitors of apoptosis and caspase activation. The effect of UF on apoptosis suggested the protection of cells via Bax-mediated mitochondrial inhibition of caspase activation on prostate cancer cells, for instance DU145 and PC3. The results confirmed the downregulation of inhibitors of apoptosis and upregulation of Bax expression. It can be easily understood that this could be translated into apoptosis and caspase activation

observed in the UF-treated group. However, the individual treatment with UF did not show any effects [14,19]. Moreover, several studies showed that the downregulation of caspase expression was correlated well with PCA.

In conclusion, UF was found to be more effective in inducing apoptosis and cell cycle arrest in both early and late stage prostate cancer cells. UF was suggested to induce cell cycle arrest, caspase activation and enhanced Bax expression via NF- κ B-independent pathways. Prominently, UF confirmed the proapoptotic effects, which appear to be tumor cell-specific. However, future *in vivo* studies are needed to scrutinize the effect of UF on the prostate cancer cells to confirm the present results.

Conflict of interests

The authors declare no conflict of interests.

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