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Influence of ellagic acid on prostate cancer cell proliferation: A caspase-dependent pathway

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ABSTRACT

Objective: To evaluate the effect of allagic acid treatment on the cell viability of human prostate cancer cells. **Methods:** Ellagic acid (10-100 mol/L) treatment (48 h) of human prostate carcinoma PC3 cells was found to result in a dose-dependent inhibition of cell growth and apoptosis of PC3 cells as assessed by MTT assay, western blotting, flow cytometry and confocal microscopy. **Results:** We observed that ellagic acid treatment of PC3 cells resulted in a dose dependent inhibition of cell growth/cell viability. This ellagic acid caused cell growth inhibition was found to be accompanied by induction of apoptosis, as assessed by the cleavage of poly (ADP-ribose) polymerase (PARP) and morphological changes. Further, induction of apoptosis accompanied a decrease in the levels of antiapoptotic protein Bcl-2 and increase in proapoptotic protein Bax, thus shifting the Bax: Bcl-2 ratio in favor of apoptosis. Ellagic acid treatment of PC3 cells was also found to result in significant activation of caspases, as shown by the dose dependent decrease in the protein expression of procaspase-3, -6, -8 and -9. This ellagic acid-mediated induction of apoptosis was significantly (80%-90%) inhibited by the caspase inhibitor N-benzyloxycarbonyl-Val-Ala-Asp (OMe)-fluoromethylketone (Z-VAD-FMK). Thus these data suggested an essential role of caspases in ellagic acid-mediated apoptosis of PC3 cells. **Conclusions:** It is tempting to suggest that consumption of tropical pigmented fruits and vegetables could be an effective strategy to combat prostate cancer.

1. Introduction

Despite recent advancements in surgery and therapy, prostate cancer remains the most common cancer. Next only to lung cancer, it is the second leading cause of cancer-related deaths in males[1]. Prostate cancer is generally detected in men over 50 years of age, usually at an advanced stage of the disease. Because of unsatisfactory outcomes associated with treating advanced cases of prostate cancer, there is a need to develop novel preventive approaches to control this disease. One such preventive approach is through chemoprevention by using naturally occurring dietary substances[2]. Epidemiological evidences strongly suggest that dietary habits such as the use of fruits and vegetables may have significant impact on the development of prostate cancer. Pigmented and tropical fruits and vegetables have been shown to contain many polyphenols, which contribute to their strong antioxidant and anti-inflammatory properties[3]. Among the polyphenols, ellagic acid (C14H6O8) is gaining considerable popularity on the fast-expanding market of food supplements as they seem to possess potentially beneficial effects against various diseases[4].

Ellagic acid is a polyphenolic compound present in fruits and berries such as pomegranates, strawberries, raspberries and blackberries. It has anticarcinogenic, antioxidant and

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antifibrosis properties[5-8]. The anticarcinogenic effect of ellagic acid was shown in several types of cancers including skin, esophageal, and colon cancers[8,9]. However, the effects of ellagic acid on prostate cancer have not been well studied. Furthermore, the mechanisms mediating anticancer effect of ellagic acid, in general, remain unknown.

Earlier we have reported the chemopreventive and chemotherapeutic effects of pomegranate juice against prostate cancer[10]. In this study, we show that ellagic acid might be the main polyphenol in the pomegranate that induces apoptosis and cell growth inhibition of highly aggressive human prostate cancer PC3 cells in an *in vitro* system.

2. Materials and methods

2.1. Cell line and reagents

PC-3 cells were obtained from American Type Culture Collection (Manassas, VA, USA) and were cultured in RPMI 1640 medium supplemented with 5% heat-inactivated fetal bovine serum and 1% antibiotic penicillin and streptomycin. The cells were maintained under standard cell culture conditions at 37 ℃ and 5% CO_2 in a humid environment.

Ellagic acid was purchased commercially from SIGMA chemicals (St. Louis, MO, USA). Antibodies to procaspases-3, -6, -8 and -9, were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Active caspases -3,-6,-8 and -9, PARP (85 kD) and Bcl-2 antibodies were procured from Cell Signaling Technology (Beverly, MA, USA). PARP (116 kD) and Bax antibodies were purchased from Upstate Biotechnology (Lake Placid, NY, USA). The general caspase inhibitor Z-VAD-FMK was obtained from R & D Systems, Inc. (Minneapolis, MN, USA).

Secondary horseradish peroxidase-conjugated antibodies were purchased from Amersham (Arlington Heights, IL, USA). Anti-mouse or anti-rabbit secondary antibody horseradish peroxidase conjugate was obtained from Amersham Pharmacia Life Sciences. The Bio-Rad DC Protein Assay Kit was purchased from Bio-Rad. Novex precast Tris-Glycine gels were obtained from Invitrogen. The Annexin-V-FLUOS Staining Kit was purchased from Roche (Indianapolis, IN, USA). The APO-BRDU kit for quantification of apoptosis by flowcytometry was from Phoenix Flow Systems (San Diego, CA, USA).

2.2. Treatment of cells

Cells were grown to 50%-70% confluence and then treated with freshly prepared ellagic acid (10-100 μM/L) dissolved in DMSO (final concentration 0.1% v/v) for 48 hours for cell growth and apoptosis assays. For caspase-inhibition experiments, the cells were incubated with Z-VAD-FMK, a general caspase inhibitor, at the desired concentration, for 4 hours before the addition of ellagic acid.

2.3. Cell viability

The effect of ellagic acid on the viability of cells was determined by 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazoliumbromide assay. The cells were plated at 1×10^4 cells per well in 200 μL of complete culture medium containing 10-100 μmol/L concentrations of ellagic acid in 96-well microtiter plates for 48 h. After incubation for specified times at 37 ℃ in a humidified incubator, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazoliumbromide (5 mg/mL in PBS) was added to each well and incubated for 2 h, after which the plate was centrifuged at 1 800 × g for 5 min at 4 ℃. The absorbance was recorded on a microplate reader at the wavelength of 540 nm. The effect of ellagic acid on growth inhibition was assessed as percent cell viability where DMSO-treated cells were taken as 100% viable. DMSO at the concentrations used was without any effect on cell viability.

2.4. Detection of apoptosis by confocal microscopy

The annexin-V-FLUOS staining kit was used for the detection of apoptosis following the vendor's protocol. This kit uses a dual-staining protocol in which the apoptotic cells are stained with annexin-V (green fluorescence), and the necrotic cells are stained with propidium iodide (PI; red fluorescence). Cells were grown to about 70% confluence and then treated with ellagic acid (10-100 μM/L) for 48 hours. The fluorescence was detected by a Ziess 410 confocal microscope.

2.5. Quantification of apoptosis by flow cytometry

For quantification of apoptosis, the cells were grown at a density of 1×10^6 cells in 100 mm culture dishes and were treated with varying doses of the ellagic acid and caspase inhibitor for 48 hours. The cells were trypsinized, washed with PBS, and were processed for labeling with fluorescein-tagged deoxyuridine triphosphate nucleotide and propidium iodide by the use of an APO-DIRECT apoptosis kit. The labeled cells were analyzed by flow cytometry.

2.6. Western blot analysis

The cells were harvested after treatment for 48 hours, homogenized in appropriate lysis buffer to prepare cell lysates[11]. Appropriate amount of protein (40 μg) was resolved over 12% Tris-Glycine polyacrylamide gel and then transferred onto the nitrocellulose membrane. The blots were blocked using 5% non-fat dry milk and probed using appropriate primary antibody and the secondary horseradish peroxidase (HRP) conjugate. The proteins were detected by chemiluminescence.

3. Results

3.1. Ellagic acid inhibits the growth of PC3 cells

Our first aim was to evaluate the effect of ellagic acid treatment on the cell viability of human prostate cancer cells. Therefore, using human prostate carcinoma PC3 cells, we first evaluated the effect of ellagic acid on the growth of these cells by 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazoliumbromide assay (MTT assay). Ellagic acid treatment to PC3 cells resulted in a significant dose-dependent inhibition of cell growth (Figure 1). The IC_{50} value at 48 hours post treatment with ellagic acid for PC3 cells was 80 μ mol/L.

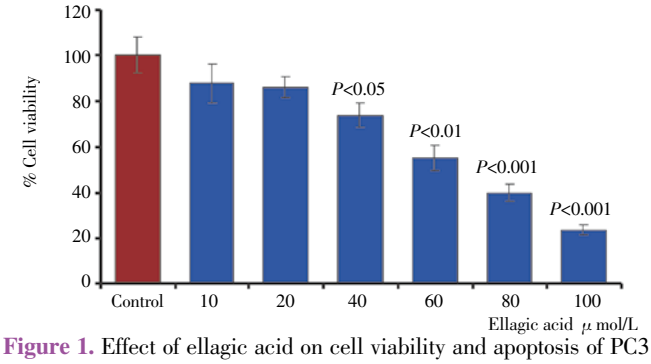

Figure 1. Effect of ellagic acid on cell viability and apoptosis of PC3 cells.

PC3 cells were treated with vehicle alone (0.1% DMSO) and specified concentrations of ellagic acid in 0.1% DMSO for 48 h, and cell viability was determined by MTT assay as detailed in Materials and Methods. The values are represented as the percent viable cells, with vehicle-treated cells regarded as 100% viable. Data shown are mean ± SD of three separate experiments in which each treatment was repeated in 10 wells.

3.2. Ellagic acid induces apoptosis in PC3 cells as assessed by fluorescence microscopy

To access whether ellagic acid-mediated decrease in cell growth is due to induction of apoptosis, we conducted Annexin V and propidium iodide stainings in ellagic acid-treated cells. We used this method because it identifies the apoptotic (green fluorescence) as well as necrotic (red fluorescence) cells. Data showed a significant induction of apoptosis by ellagic acid which was evident from the significant enhancement in Annexin V staining (Figure 2).

3.3. Ellagic acid brings on PARP cleavage

During the induction of apoptosis, DNA fragmentation leads to activation of Poly (ADP-ribose) polymerase (PARP); therefore, cleavage of PARP protein is considered as an important biomarker of apoptosis. Immunoblot analysis showed a significant dose-dependent increase in cleaved PARP in cells treated with ellagic acid (Figure 3).

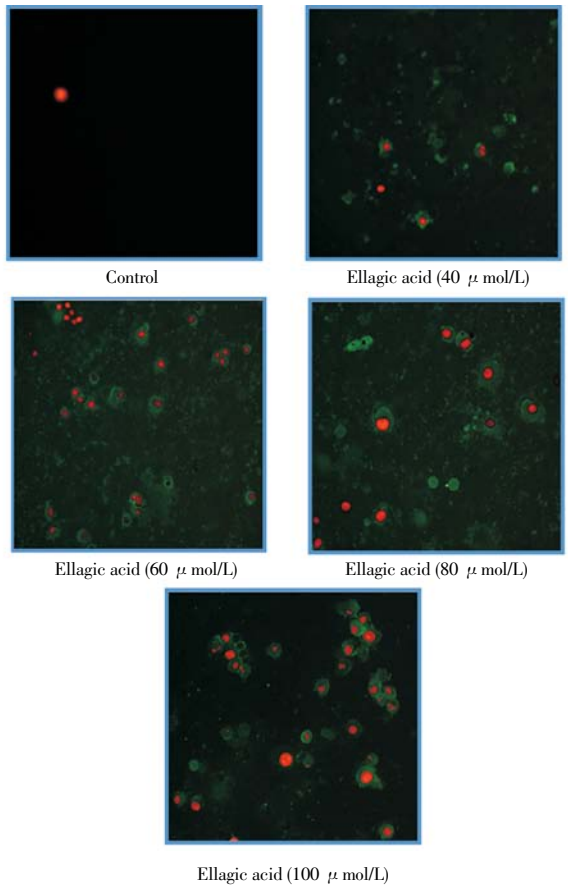

Figure 2. Representative micrographs of PC3 cells undergoing apoptosis induced by treatment with specified concentrations of ellagic acid for 48 h as assessed by fluorescence microscopy.

Green fluorescence of Annexin V staining represents the cells undergoing apoptosis and red fluorescence of propidium iodide shows the cells undergoing either necrosis or late apoptosis as detected with a Zeiss Axiovert 100 microscope.

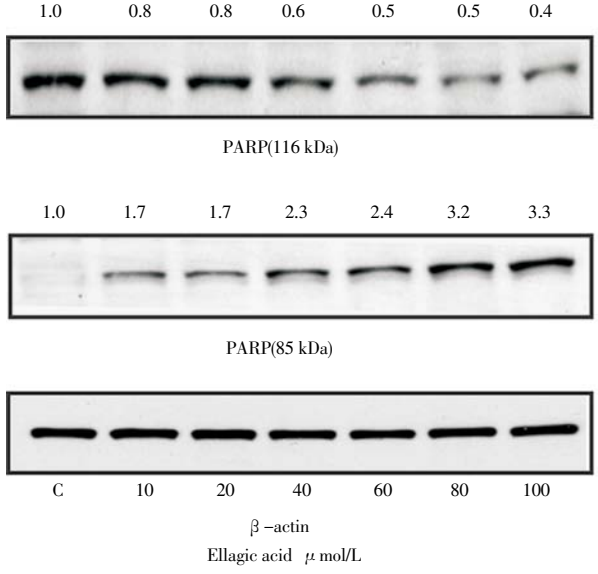

Figure 3. Effect on cleavage of PARP.

The cells were treated with vehicle only or specified concentrations of ellagic acid for 48 h and harvested, and cell lysates were prepared. The data are representative of three independent experiments with similar results. The values above the blots represent the change in the protein expression of the bands normalized to β -actin where vehicle-treated cells are represented as 1.0.

3.4. Ellagic acid alters Bax and Bcl-2 protein expression in PC3 cells

Bcl2 forms a heterodimeric complex with the apoptotic Bax protein, thereby neutralizing its apoptotic effects. Therefore, the ratio of Bax/Bcl2 is often considered as a decisive factor in determining whether cells will undergo death or survive. We observed that ellagic acid treatment of cells resulted in a decrease in Bcl2 expression with a concomitant increase in the protein level of Bax (Figure 4). This resulted in a substantial increase in Bax/Bcl2 ratio, which favors apoptosis. Taken together, these findings suggest that cleavage in PARP protein, up-regulation of Bax, and down-regulation of Bcl2 may collectively form a molecular basis for the apoptotic action of ellagic acid.

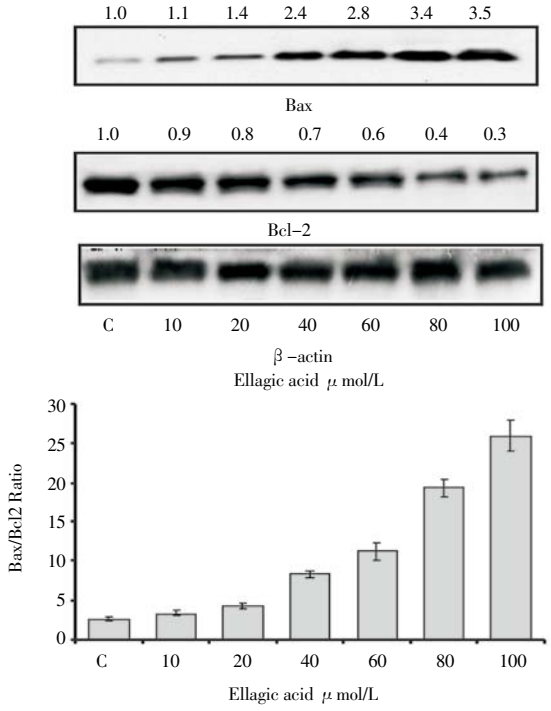

Figure 4: Effect of ellagic acid treatment of PC3 cells on protein expression of Bax and Bcl2 and the Bax/Bcl2 ratio.
The cells were treated with vehicle only or specified concentrations of ellagic acid for 48 h and harvested, and cell lysates were prepared. The data are representative of three independent experiments with similar results. The values above the blots represent the change in the protein expression of the bands normalized to β -actin where vehicle-treated cells are represented as 1.0.

3.5. Ellagic acid stimulates apoptosis through activation of caspases in PC3

In most cancer cells, caspases are present in the pro-forms (inactive) and require site-specific cleavage of the protein to become active and participate in the process of apoptosis. To test whether caspases are involved in apoptosis induction by ellagic acid, we first evaluated the protein levels of procaspases and active caspases in ellagic acid-treated cells. Data presented showed a significant and progressive increase in the levels of initiator caspases (active caspase-8 and caspase-9) as well as effector caspases (active caspase-3 and caspase-6) proteins in ellagic acid-treated cells (Figure 5 and 6).

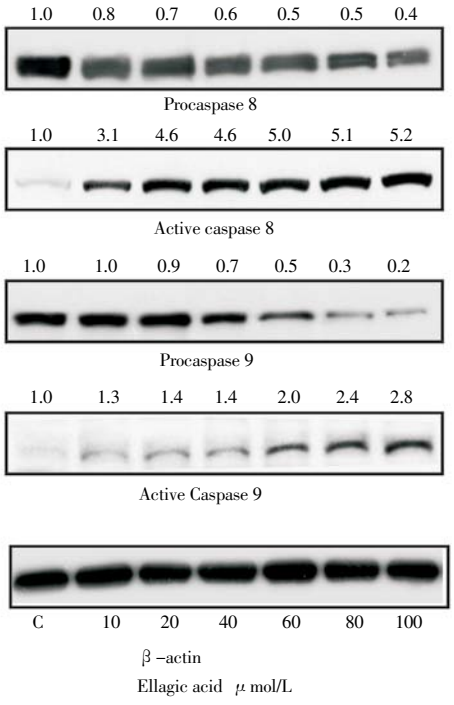

Figure 5. Effect of ellagic acid on initiator caspases in PC3 cells.
PC3 cells were treated with vehicle alone (0.1% DMSO) or specified concentrations of ellagic acid in 0.1% DMSO for 48 h as detailed in Materials and methods.
Protein levels of procaspase-8, procaspase-9, active caspase-8, and caspase-9 in PC3 cells were determined by immunoblot analysis. The data are representative of three independent experiments with similar results. The values above the blots represent the change in the protein expression of the bands normalized to β -actin where vehicle-treated cells are represented as 1.0.

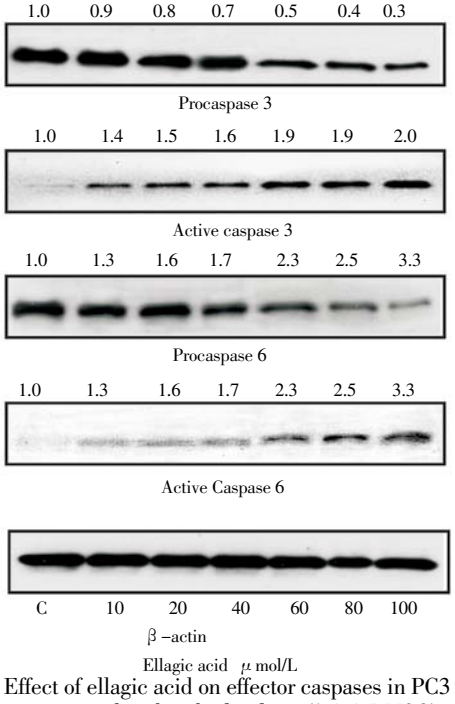

Figure 6. Effect of ellagic acid on effector caspases in PC3 cells.
PC3 cells were treated with vehicle alone (0.1% DMSO) or specified concentrations of ellagic acid in 0.1% DMSO for 48 h as detailed in Materials and Methods. Protein levels of procaspase-3, procaspase-6, active caspase-3, and caspase-6 in PC3 cells were determined by immunoblot analysis. The data are representative of three independent experiments with similar results. The values above the blots represent the change in the protein expression of the bands normalized to β -actin where vehicle-treated cells are represented as 1.0.

To test whether ellagic acid induces apoptosis via activation of caspases, we used a general caspases inhibitor, Z-VAD-FMK. Ellagic acid (80 μ mol/L)-treated cells exhibited 29.6% terminal deoxyribonucleotidyl transferase-mediated dUTP nick end labeling-positive cells, which was significantly reduced to 9.45% with the treatment of cells with Z-VAD-FMK (Figure 7).

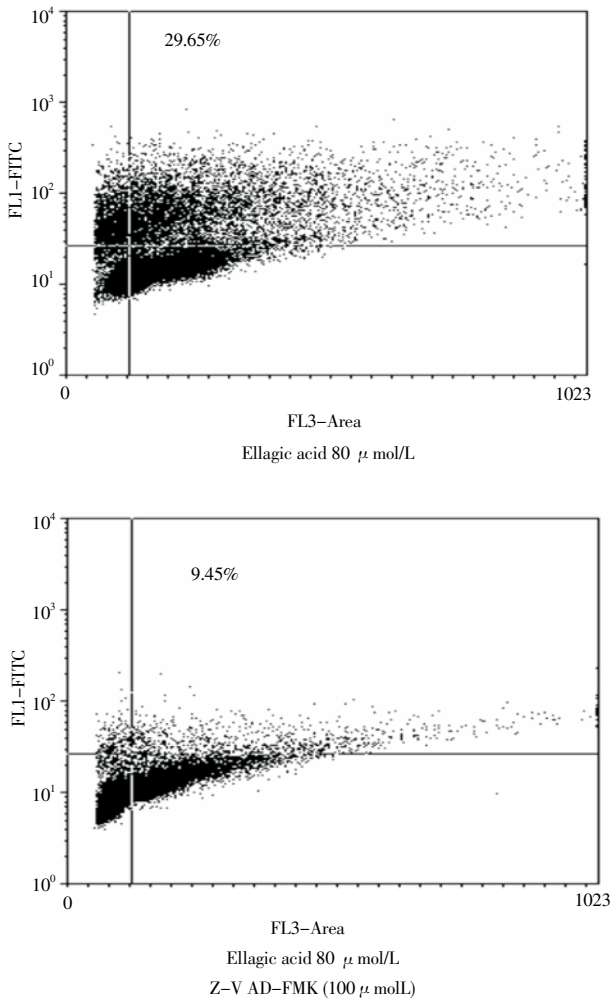

Figure 7. Inhibition of caspase activity as assessed by flow cytometry. PC3 cells were treated with 10 μ mol/L concentration of the general caspase inhibitor Z-VAD-FMK for 4 h, followed by the treatment with the indicated dose of ellagic acid for 48 h. Cells showing dUTP fluorescence above that of control population, as indicated by the line in each histogram, are considered as apoptotic cells.

Immunoblot analysis also showed that ellagic acid-induced activation of caspases-3, 6, 8 and-9 was significantly reduced after caspase inhibitor treatment (Figure 8). These results suggest that induction of caspases may be a possible mechanism by which ellagic acid induces apoptosis in PC3 cells.

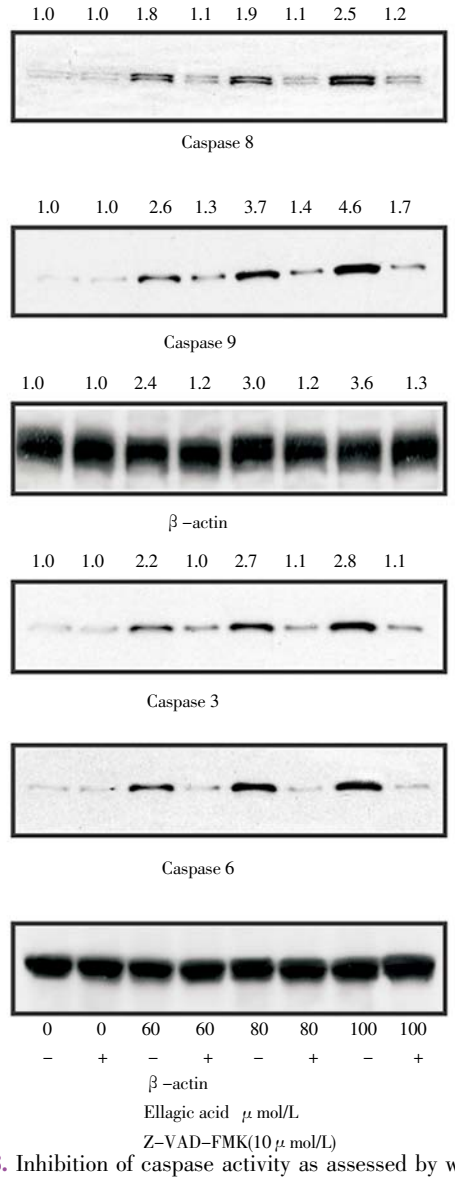

Figure 8. Inhibition of caspase activity as assessed by western blot analysis.
PC3 cells were treated with 10 μ mol/L concentration of the general caspase inhibitor Z-VAD-FMK for 4 h, followed by the treatment with the indicated doses of ellagic acid for 48 h. The data are representative of three independent experiments with similar results. The values above the blots represent the change in the protein expression of the bands normalized to β -actin where vehicle-treated cells are represented as 1.0.

4. Discussion

Recently, chemoprevention and other intervention approaches using naturally occurring agents have come out as a promising choice that could prevent or slow the tumor growth thus improve the quality of life of prostate cancer patients. In this regard, several fruit- and vegetable-derived chemopreventive agents have been reported that induce apoptosis in cancer cells in both in vitro and in vivo systems[12,13]. We have previously reported that pomegranate juice brings on apoptosis in prostate cancer cells and inhibits tumor growth in athymic nude mice[10].

In the present study we showed that ellagic acid might be the main potential polyphenol in the pomegranate. Here we observed the antiproliferative effect of ellagic acid on PC3 cells. We found that the primary mode of ellagic acid-mediated inhibition of tumor growth is through the induction of apoptosis.

Because caspases have been shown to be involved in apoptosis[14,15] via activation of downstream effector molecules such as PARP[16,17], we found that the induction of apoptosis by ellagic acid is governed primarily by the activation of caspases because pretreatment with Z-VAD-FMK, a caspase inhibitor, significantly prevented ellagic acid-mediated apoptosis.

Members of the Bcl-2 family of proteins are critical regulators of the apoptotic pathway. Bcl-2 is an upstream effector molecule in the apoptotic pathway and is identified as a potent suppressor of apoptosis[18]. Bcl-2 is found at inappropriately high levels in more than half of all human tumors[19,20]. Bcl-2 has been shown to form a heterodimer complex with the proapoptotic member Bax, thereby neutralizing its proapoptotic effects. Therefore, the ratio of Bax/Bcl-2 is a decisive factor and plays an important role in determining whether cells will undergo death or survival. In our study, ellagic acid treatment of PC3 cells for 48 h was found to result in a decrease in Bcl-2 protein expression with an increase in the protein expression of Bax Importantly, in ellagic acid-treated cells, the ratio of Bax to Bcl-2 was found to be altered in favor of apoptosis. Our results thus suggest that up-regulation of Bax and down-modulation of Bcl-2 may be another molecular mechanism through which ellagic acid induces apoptosis.

Thus this present study is showing the effect of ellagic acid in inhibiting human prostate cancer cell growth in in vitro model via activation of caspase pathway. There is an increasing interest in the use of natural products for cancer treatments. Based on the present findings, it is tempting to suggest that ellagic acid could be developed as a potential anticancer agent against human prostate cancer cells.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgements

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References

[1] Jemal A, Siegel R, Ward E, Xu J. Cancer statistics, 2010. *CA Cancer J Clin* 2010; **60**: 277-300.

[2] Syed DN, Khan N, Afaq F, Mukhtar H. Chemoprevention of prostate cancer through dietary agents: progress and promise. *Cancer Epidemiol Biomarkers Prev* 2007; **16**: 2193-203.

[3] Khan N, Afaq F, Mukhtar H. Cancer chemoprevention through dietary antioxidants: progress and promise. *Antioxid Redox Signal* 2008; **10**: 475-510.

[4] Bell C, Hawthorne S. Ellagic acid, pomegranate and prostate cancer. *J Pharm Pharmacol* 2008; **60**: 139-144.

[5] Mukhtar H, Das M, Khan WA, Wang ZY, Bik DP, Bickers DR. Exceptional activity of tannic acid among naturally occurring plant phenols in protecting against 7,12-dimethylbenz(a)anthracene-, benzo(a)pyrene-, 3-methylcholanthrene-, and N-methyl-N-nitrosourea-induced skin tumorigenesis in mice. *Cancer Res* 1988; **48**:2361-2365.

[6] Thresiamma KC, Kuttan R. Inhibition of liver fibrosis by ellagic acid. *Indian J Physiol Pharmacol* 1996; **40**:363-366.

[7] Osawa T, Ide A, Su JD, Namiki M. Inhibition of lipid peroxidation by ellagic acid. *J Agric Food Chem* 1987; **35**:808-812.

[8] Stoner GD, Gupta A. Etiology and chemoprevention of esophageal squamous cell carcinoma. *Carcinogenesis* 2001; **22**:1737-1746.

[9] Larrosa M, Tomas-Barberan FA, Espin JC. The dietary hydrolysable tannin punicalagin releases ellagic acid that induces apoptosis in human colon adenocarcinoma Caco-2 cells by using the mitochondrial pathway. *J Nutr Biochem* 2006; **17**:611-625.

[10] Malik A, Afaq F, Sarfaraz S, Adhami VM, Syed DN, Mukhtar H. Pomegranate fruit juice for chemoprevention and chemotherapy of prostate cancer. *Proc Natl Acad Sci U S A* 2005; **102**: 14813-14818.

[11] Saleem M, Afaq F, Adhami VM, Mukhtar H. Lupeol modulates NF-κB and PI3K/Akt pathways and inhibits skin cancer in CD-1 mice. *Oncogene* 2004; **23**: 5203-5214.

[12] Saleem M, Kweon MH, Yun JM. A novel dietary triterpene Lupeol induces fas-mediated apoptotic death of androgen-sensitive prostate cancer cells and inhibits tumor growth in a xenograft model. *Cancer Res* 2005; **65**: 11203-11213.

[13] Siddiqui IA, Zaman N, Aziz MH. Inhibition of CWR22R v 1 tumor growth and PSA secretion in athymic nude mice by green and black teas. *Carcinogenesis* 2006; **27**: 833-839.

[14] Gupta S, Hastak K, Afaq F, Ahmad N, Mukhtar H. Essential role of caspases in epigallocatechin-3-gallate-mediated inhibition of nuclear factor κB and induction of apoptosis. *Oncogene* 2004; **23**: 2507-2522.

[15] Hafeez BB, Ahmed S, Wang N, Gupta S, Zhang A, Haqqi TM. Green tea polyphenols-induced apoptosis in human osteosarcoma SAOS-2 cells involves a caspase-dependent mechanism with down-regulation of nuclear factor-κB. *Toxicol Appl Pharmacol* 2006; **216**: 11-19.

[16] Nicholson DW. Caspase structure, proteolytic substrates, and function during apoptotic cell death. Cell Death Differ 1999; **6**: 1028-1042.

[17] Zimmermann KC, Bonzon C, Green DR. The machinery of programmed cell death. *Pharmacol Ther* 2001; **92**: 57-70.

[18] Hockenbery DM, Oltvai ZN, Yin XM, Milliman CL, Korsmeyer SJ. Bcl-2 functions in an antioxidant pathway to prevent apoptosis. *Cell* 1993; **75**: 241-251.

[19] Reed JC. Regulation of apoptosis by bcl-2 family proteins and its role in cancer and chemoresistance. *Curr Opin Oncol 1*995; **7**: 541-546.

[20] Revelos K, Petraki C, Gregorakis A, Scorilas A, Papanastasiou P, Koutsilieris M. Immunohistochemical expression of Bcl2 is an independent predictor of time-to-biochemical failure in patients with clinically localized prostate cancer following radical prostatectomy. *Anticancer Res* 2005; **25**: 3123-3133.