Food Sci. Biotechnol. 24(5): 1887-1892 (2015)
DOI 10.1007/s10068-015-0247-0

Food Science and Biotechnology

Effect of Pomegranate Peel Polyphenols on Human Prostate Cancer PC-3 cells *in vivo*

Gui-Zhi Ma, Chun-Mei Wang[1], Li Li[2], Nan Ding[1], and Xiao-Li Gao*

College of Pharmacy, Xinjiang Medical University, Urumqi 830011, China
[1]*The First Teaching Hospital of Xingjiang Medical University, Urumqi 830011, China*
[2]*Beijing Key Laboratory of Plant Resources Research and Development, Beijing Technology and Business University, Beijing 100048, China*

Received December 17 2014
Revised March 24 2015
Accepted March 25 2015
Published online October 31 2015

*Corresponding Author
Tel: +86-13609905058
Fax: 0991-4366520
E-mail: xli_g@sina.com

pISSN 1226-7708
eISSN 2092-6456

Abstract *Punica granatum* possesses strong antitumor properties. In this study, a nude mouse model with a subcutaneous xenograft of human prostate cancer cells (PC-3) was established to observe the *in vivo* antiproliferative and apoptotic effects of pomegranate peel polyphenols (PP). Levels of the cytokines tumor necrosis factor α (TNF-α) and vascular endothelial growth factor (VEGF) were detected by enzyme-linked immunosorbent assay. Quantitative analysis of ellagic acid, gallic acid, and punicalagin in active fraction was conducted using reverse phase high-performance liquid chromatography with a photo-diode array (HPLC/PDA). PP decreased tumor volume and weight in tumor-bearing nude mice, and significantly increased the rate of apoptosis (p<0.05). In addition, PP increased TNF-α and decreased VEGF in the serum (p<0.05). Ellagic acid (201.3±3.544 mg/g), gallic acid (8.917±0.274 mg/g), and punicalagin (407.0±12.05 mg/g) were the main effectors of the anti-tumor activity.

Keywords: pomegranate peel polyphenols, prostate cancer, TNF-α, VEGF, HPLC/PDA

Introduction

Prostate cancer (CaP) is one of the most common cancers in European and American males and has a high mortality rate. The number of new cases in the United States in 2007 was projected to be 218,890, with 27,000 deaths expected from the disease. This estimate suggests that about 1 in 6 men will be diagnosed with CaP during his lifetime, and 1 in 34 men diagnosed with CaP will die of the disease. Similar trends have been observed in most industrialized Western countries (American Cancer society. Cancer Facts and Figures. 2007. www.cancer.org). One approach to decreasing the incidence of CaP is chemoprevention, a means of cancer control in which the occurrence of the disease can be entirely prevented, slowed, or reversed by the administration of one or more naturally occurring and/or synthetic compounds (1-4).

Epidemiological studies suggest that consumption of a phytochemical-rich diet that includes fruits and vegetables is associated with a reduced risk of cancer. Fresh and processed fruits and vegetable products contain high levels of a diverse range of phytochemicals, of which polyphenols make up a large proportion. A number of phytochemicals, including resveratrol from grapes and red wine, sulforaphane from broccoli and other cruciferous vegetables, organosulfides from garlic and other *Allium* species, limonene and perillyl alcohol from the lipid fraction of citrus peels, isoflavones (such as genistein and daidzein) and enterodiols from soy and flax proteins, catechins from green tea, and lycopene from tomatoes have been proposed as potential chemoprevention agents based on animal and laboratory evidence of antitumor effects (3,4). Pomegranate, as a rich source of polyphenolic compounds including anthocyanins and hydrolysable tannins, possesses stronger antioxidant activity than green tea and red wine do (5).

Pomegranate fruits, beverages, and related products are frequently highlighted for their putative beneficial health effects on arthritis, diabetes, cardiovascular disease, prostate cancer, and other medical conditions (6,7). Recent research has shown that pomegranate extracts selectively inhibit the growth of breast, prostate, colon, and lung cancer cells in culture (8). The tannin ellagic acid (*EA*), the phenolic acid caffeic acid (*C*), the estrogenic flavone luteolin (*L*), and the conjugated trienoic fatty acid punicic acid (*P*), are compounds with known anti-cancer activity that are found in substantial amounts in the peel, juice, and seed oil of the pomegranate fruit (*Punica granatum*) (9-11). Various preparations of pomegranate, in the form of oils, fermented juice polyphenols, and pericarp polyphenols, were tested on human CaP cell growth both *in vitro* and *in vivo*. Each preparation inhibited growth of human CaP, LNCaP, PC-3, and DU 145 cells, whereas normal prostate epithelial cells were significantly less affected (3).

In early research, the authors established a preparation method for the isolation of active polyphenols from pomegranate peel (PP). The total content of active constituents in the final extract was

greater than 50% (w/w). In this study, we evaluated the effects of PP using a PC-3 nude mouse xenograft model.

Materials and Methods

Materials

Chemical and Reagents: Flutamide was purchased from Sigma-Aldrich (St. Louis, MO, USA). Fetal bovine serum (FBS), Dulbecco's modified Eagle's medium (DMEM), and trypsin were purchased from GE Healthcare (Little Chalfont, UK). The TUNEL cell apoptosis detection kit was purchased from Roche (Basel, Switzerland). The vascular endothelial growth factor (VEGF) enzyme-linked immunosorbent assay (ELISA) kit and tumor necrosis factor-α (TNF-α) ELISA kit were purchased from eBioscience (San Diego, CA, USA). HPLC grade methanol was purchased from Merck (Darmstadt, Germany). Ultra-pure water (18.2 MΩ) was prepared with a Milli-Q water purification system (Millipore, Bedford, MA, USA).

Ellagic acid (≥95%) was purchased from Sigma-Aldrich, gallic acid (≥95%) was purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China), and punicalagin (98%) was purchased from Tianjin Party Technology Co., Ltd. (Tianjin, China). The structures of these compounds are shown in Fig. 1. For the quantitative analysis, standard solutions of ellagic acid (0.171 mg/mL), gallic acid (0.402 mg/mL), and punicalagin (0.682 mg/mL) were prepared. Each standard was dissolved in methanol. The standard solutions were stored in dark glass bottles at 4°C. The working standard solutions were freshly prepared before injection by diluting suitable amounts of the standard solutions with methanol. The standard solutions were filtered through a 0.45 μm filter and injected (10.0 μL) into the HPLC for analysis. A Prominence LC-20AB (Shimadzu, Kyoto, Japan) was used to perform the quantitative analysis of PP.

Cell culture: PC-3 human prostate cancer cells were purchased from the Cell Bank of the Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences. PC-3 is a hormone-independent epithelial prostate cancer cell line. Cells were cultured at 37°C with 5% CO_2 in RPMI 1640 medium supplemented with 10% fetal bovine serum, penicillin (100 IU/mL), and streptomycin (100 mg/mL), and used at the time points indicated in the results.

Animals Male BALB/cA-nu mice (18-22 g) aged between 3 and 4 weeks were obtained from Hunan Slack King Laboratory Animal Co., Ltd., Changsha, China (license number: scxk [Hunan] 2009-0004). The animals were housed under standard conditions (temperature, 25±1°C; relative humidity, 50-55%; 12 h/12 h light/dark cycle). The animals were given standard laboratory feed and water ad libitum.

Preparation of PP The pomegranate peel (0.5 kg) was crushed, passed through an 80 mesh (0.18 mm) screen, and extracted with ethanol (60%, v/v) at a ratio of 1:24 for solid material to liquid material. The extraction was filtered and concentrated to 20.8 mg/mL. The solution was added to a macroporous resin column, and successively eluted with water and ethanol (50%, v/v). The ethanolic extract (50%, v/v) was collected and lyophilized to a PP powder, which was used for the activity estimations. In addition, PP (0.1 g) was extracted with methanol (50 mL) and diluted to a reasonable concentration for the quantification of ellagic acid, gallic acid, and punicalagin.

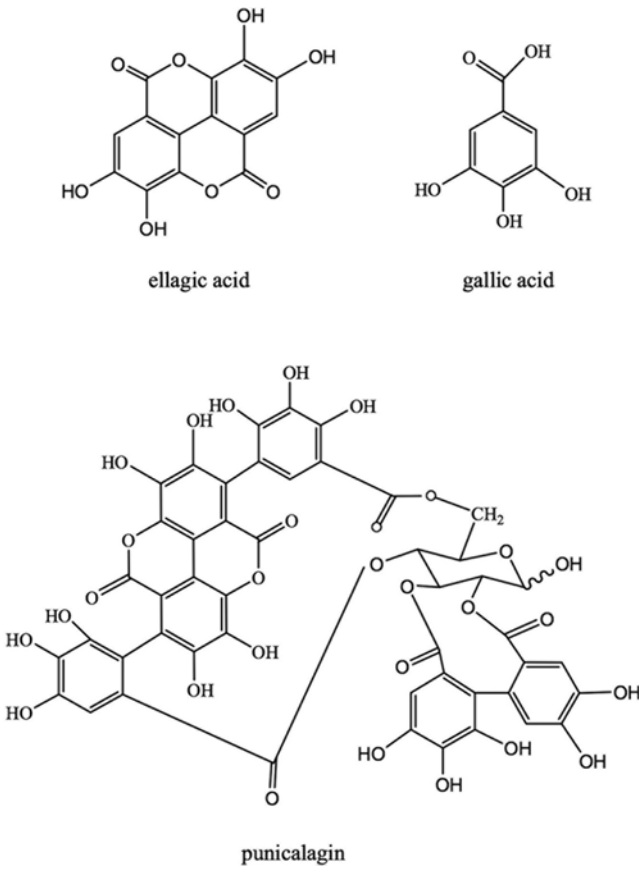

Fig. 1. The structures of the standard compounds.

Xenograft model PC-3 cells were collected in the logarithmic phase and adjusted to a concentration of 1×10^7/mL, and 0.2 mL of the cell culture was aseptically injected into the abdomen of each BALB/cA-nu mouse. When tumors became palpable 2 weeks after tumor cell injection, mice were orally administered either PP (80, 112, or 187 mg/kg) or vehicle. Nude mice with tumors (100 to 300 mm^3) were selected for experiments and randomly grouped into the negative control group (physiological saline, NS), positive control group (flutamide, 40 mg/kg), low dosage group (80 mg/kg), medium dosage group (112 mg/kg), or high dosage group (187 mg/kg) (n=8, mice per group). The mice were treated by intragastric administration every day for 17 consecutive days. The long diameter (a) and short diameter (b) of the tumors were measured every 3-4 days. Blood samples were collected via retro-orbital blood collection 24 h after

the last treatment. Xenografts from the nude mice were removed and weighed to calculate the rate of tumor inhibition. The tumor inhibition rate (%) for each group was calculated as:

$$\text{Tumor inhibition rate (\%)}=(1-TW_{treated}/TW_{control})\times 100$$

where $TW_{treated}$ was the average tumor weight of the treated group and $TW_{control}$ was the average tumor weight of the negative control group.

Moreover, the hearts, livers, and kidneys of nude mice were collected for pathological studies. The tumor volume was calculated ($v=(a\times b^2)/2$) and the tumor growth curve was obtained (12).

Analysis of tumor tissue by light microscopy Tumors were removed and anatomized while avoiding central areas of liquefactive necrosis and the peripheral region of the tumor (13). Several tissue samples were obtained from both sides of the tumor and immediately fixed in 10% formaldehyde solution. The tissue sections were rinsed, followed by gradient ethanol dehydration, xylene elution, embedding, hand trimming, and ultrathin sectioning. The sections were stained with hematoxylin-eosin (HE) and sealed with neutral glue. The stained slides were photographed under a light microscope.

Xenograft apoptosis in nude mice The terminal deoxynucleotidyl transferase-mediated dUTP biotin nick end labeling (TUNEL) method was used to detect apoptosis in xenografts from nude mice. Sections embedded in paraffin were deparaffinized, rehydrated, and subjected to antigen retrieval using 0.25% (mass fraction) trypsin at 37°C for 30 min. The sections were rinsed twice with PBS and 50 μL of the TUNEL reaction mixture was added. The TUNEL reaction mixture was replaced with reagent 2 (labeling solution) for the negative control group. The reaction mix was incubated at 37°C for 60 min and rinsed 3 times with PBS. Transferring agent (POD, 50 μL) was added and the sections were incubated in a humidified chamber at 37°C for 30 min, followed by rinsing with PBS and staining with 3,3-diaminobenzidine (DAB) to measure chromogenesis. Sections were counter stained with hematoxylin, followed by dehydration, penetration, and sealing. The nuclei of apoptotic cells were brown in color, and they were smaller and more fragmented than the nuclei of normal cells. Three high power fields (light microscope, 400× magnification) were randomly selected, and the numbers of apoptotic cells and total cells were counted and used to calculate the rate of apoptosis using the following equation:

$$\text{apoptotic rate (\%)}=(N_a/N_t)\times 100$$

where N_a was the number of apoptotic cells and N_t was the total number of cells.

Estimation of VEGF and TNF-α by ELISA All procedures were performed according to the instructions from the ELISA kit manufacturers. The standard curve was prepared by plotting the optical density (OD) values over the concentrations and used to determine the contents of VEGF and TNF-α in serum from tumor-bearing mice.

Quantitation of ellagic acid, gallic acid, and punicalagin in PP The chromatographic column was an Agilent TC-C18 (2) (4.6 mm×250 mm, 5 μm) (Agilent Technologies, Santa Clara, CA, USA). The flow rate was 1.0 mL/min and the column temperature was set to 30°C. Three different elution requirements were established to quantify ellagic acid, gallic acid, and punicalagin. For ellagic acid, the detection wavelength was set at 254 nm. The mobile phase was a buffer containing acetoacetate: phosphoric acid (0.01 mol/ L):potassium dihydrogen phosphate (KDP, 0.01 mol L^{-1}):methanol (0.01:25:25:50). For gallic acid, the detection wavelength was set at 273 nm and the mobile phase was methanol: 0.05% phosphoric acid (3:97). For punicalagin, the detection wavelength was set at 370 nm and the mobile phase consisted of water: trifluoroacetic acid (99.9:0.1, v/v) (A) and methanol (B). The gradient program was 5-20% (B) from 0-10 min, 20-45% (B) from 10-25 min, 45-65% (B) from 25-35 min, and 65% (B) from 35-40 min. Peak assignments were made for each single-compound injection by comparing retention times and characteristic absorption spectra from the PDA with those of the standards. The contents were calculated using standard curves. The content of punicalagin was calculated using a standard curve drawn according to the total peak areas of the α- and β-isomers. Validation of the quantitative analysis of ellagic acid, gallic acid, and punicalagin was performed according to the guidelines of ICH Topic Q2B (14).

Data analysis The data was analyzed by one-way analysis of variance (ANOVA) using SPSS 17.0 (IBM Corp., Armonk, NY, USA). The statistical significance of the differences between each treated group and the control group were determined using Dunnett's t-test. Results of $p<0.05$ were considered statistically significant.

Results and Discussion

Inhibition of tumor growth by PP The rates of tumor inhibition in tumor-bearing mice after treatment with high, medium, or low doses of PP were 41.66, 36.57, and 31.89%, respectively, which were all significantly different from the tumor inhibition rate of the physiological saline group ($p<0.05$), as shown in Table 1. PP treatment reduced tumor weight in the tumor-bearing mice. The tumor growth curve (tumor volume over time) is shown in Fig. 2.

Histopathological examination of subcutaneous PC-3 cell xenografts in nude mice Tumor cells were densely distributed with an irregular arrangement in the negative control group. Tumor cells in the low dosage group (80 mg/kg PP) were shrunken and showed unclear intracellular boundaries with pyknotic nuclei, and fewer tumor cells were present. Laminar necrosis was also observed in isolated areas. Tumor cells in the medium dosage (112 mg/kg PP)

Table 1. Effect of pomegranate peel polyphenols on tumor inhibition ratio

Group	Dosage mg/kg/d	*n*		Tumor weight/g	Tumor inhibition Ratio/%
		Begin	End		
Control	0	8	7	0.53±0.20	-
Flutamide	40	8	7	0.28±0.14*[1)]	46.78
Low dosage	80	8	7	0.36±0.17**	31.89
Med dosage	112	8	8	0.33±0.16**	36.57
High dosage	187	8	8	0.31±0.10*	41.66

[1)]* and ** represent significant differences at $p<0.05$ and $p<0.01$, respectively.

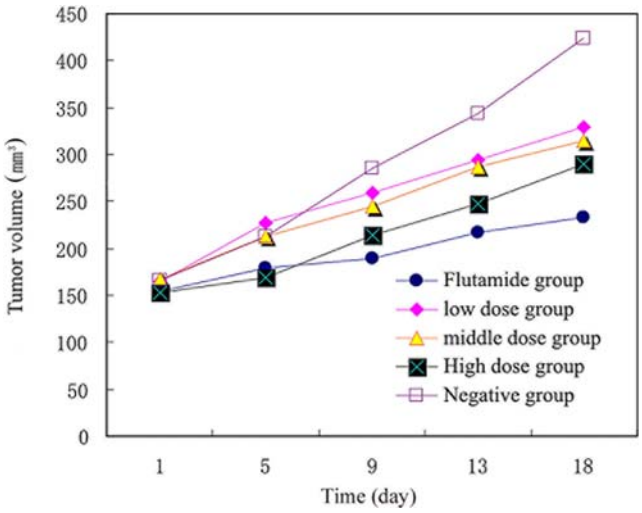

Fig. 2. Effect of anticancer effective parts of PP on tumor volume.

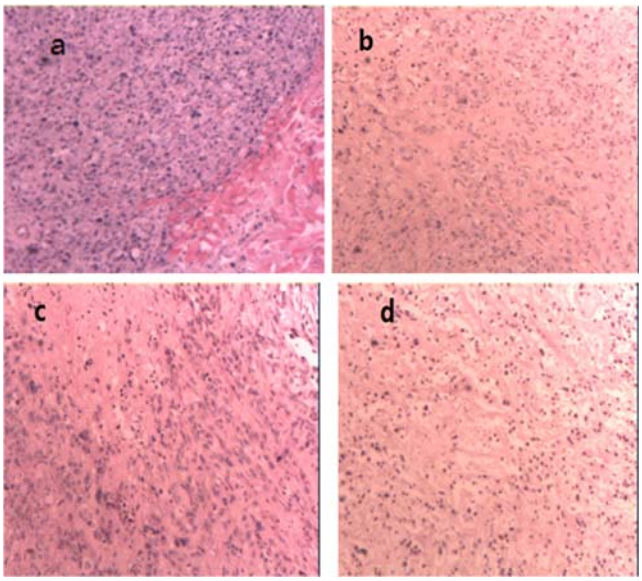

Fig. 3. Pathology of transplantation tumor tissues.

group showed cellular edema and dissolved nuclei along with some necrotic areas in the tumor cell population. Tumor cells in the high dosage group (187 mg/kg PP) showed cellular edema and dissolved nuclei. Large necrotic areas were also observed, as shown in Fig. 3.

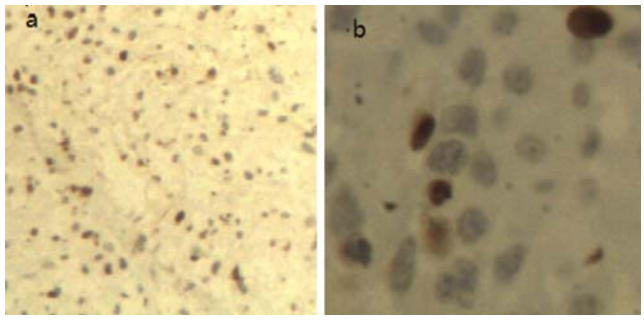

Fig. 4. Apoptosis was detected by TUNEL assay.

Toxicity of PP in tumor-bearing nude mice Nude mice treated with PP had normal body weight, feeding, and observable behavior. In the pathological study, the low dose (80 mg/kg PP) group showed no toxicity in the heart, liver, or kidney, whereas the medium dose (112 mg/kg PP) group showed acidophilic degeneration of some cardiomyocytes, partial vascular congestion in the myocardial interstitium, hemangiectasis of some liver sinusoidal endothelial cells, and infiltration of inflammatory cells. The high dose (187 mg/kg PP) group showed slight inflammatory cell infiltration in the myocardial interstitium, some dotted myocardial hemorrhage and necrosis, slight edema of epithelial cells of the renal proximal convoluted tubules with mild congestion of some glomeruli, liver sinusoidal dilatation, and inflammatory cell infiltration.

Apoptosis in PC-3 cells A large number of apoptotic cells were observed in tumor tissue from the PP-treated groups. Moreover, the nuclei were brown and relatively small or fragmented, whereas tumor tissue from the negative control group (treated with physiological saline) had fewer apoptotic cells (Fig. 4). The rate of apoptosis of each group is shown in Table 2.

TNF-α and VEGF levels in serum from tumor-bearing mice Serum TNF-α levels in the PP-treated tumor-bearing groups were significantly higher than that of the negative control group. Although all doses of PP increased serum TNF-α levels in tumor-bearing mice, the medium (112 mg/kg) and high (187 mg/kg) doses significantly reduced serum VEGF levels in tumor-bearing mice in comparison with that of the negative control group. The low dose (80 mg/kg PP) group did not

Table 2. Effect of pomegranate peel polyphenols on apoptosis index, VEGF and TNF-α contents

Group	Dosage mg/kg/d	Apoptosis index (*n*=6)	VEGF (pg/mL)	TNF-α (pg/mL)
Control	0	11.50±0.01	148.20±14.63	90.26±11.58
Positive control	40	-	140.24±15.84	144.33±18.12
Low dosage	80	19.63±1.82*[1)]	139.22±25.05	144.89±17.22
Med dosage	112	22.35±1.79*	128.61±16.98*	178.78±18.54**
High dosage	187	26.68±1.33*	105.36±18.08**	285.63±21.76**

[1)]* and ** represent significant differences at $p<0.05$ and $p<0.01$, respectively.

show a significant difference in serum VEGF level in comparison with the control group. The TNF-α and VEGF measurements are shown in Table 2.

TNF-α plays a major role in cell proliferation and differentiation, tumor angiogenesis, apoptosis, necrosis, immune regulation, lipid metabolism, and vascular endothelial cell generation. TNF-α promotes local or systemic inflammatory responses, and sepsis increases TNF-α expression, which is relatively stable during inflammation and cachexia. TNF-α promotes the expression of tumor suppressor gene p53, thus enhancing the body's anti-tumor response. ELISA was used to explore the effects of PP on serum levels of TNF-α and VEGF in tumor-bearing mice. The PP-treated groups showed significant increases in TNF-α in comparison with the negative control group. The medium and high dose PP groups showed reduced serum VEGF levels, indicating that PP might promote tumor cell apoptosis and necrosis by increasing the expression of TNF-α, perhaps by up-regulation of caspase-8 expression through binding with Fas protein, or by damaging peripheral blood vessels of solid tumors to block the supply of nutrients, which eventually leads to necrosis and suppresses tumor growth.

Primary constituents of PP Three batches of PP were tested, and the contents (mean±SD) of ellagic acid gallic acid, and punicalagin were 201.3±3.544, 8.917±0.2740, and 407.0±12.05 mg/g, respectively. The polyphenols ellagic acid, gallic acid, and punicalagin constituted 61.72 % of the PP. Punicalagin consisted of α- and β-isomers, and it was not stable and showed reciprocal transformation in the tested solution. Pomegranates are rich in polyphenols, and are known to contain gallic acid, granatin A, granatin B, ellagic acid, punicalin, punicalagin, and other compounds. Gallic acid has strong antioxidant activity and it is one of the principal bioactive ingredients in pomegranates. A previous study showed that gallic acid inhibited tumor cell growth and induced tumor cell apoptosis (15). Ellagic acid, a 2-poly derivative of gallic acid, is widely used in the fields of food, medicine, health care, and cosmetics. Ellagic acid has antioxidant, anticancer, anti-mutation, antibacterial, and antiviral effects. Punicalagin is one of the major components of pomegranate peel, and it has been isolated only from the leaves of the olive kernel tree, *Terminalia chebula*, and a few other plants. In literatures, EA was potential chemopreventive agents for prostate cancer. Treatment with non-toxic concentration of EA on androgen-independent prostate cancer, PC3 and PLS10 cells markedly reduced the motility and the invasion of the cells. EA had ability to reduce the invasiveness of prostate cancer cell lines by the modulation of MMP activity (16-18). Punicalagin has antioxidant, tumor inhibitory, and anti-atherosclerotic effects. Three different HPLC methods were established for quantitative analysis of gallic acid, ellagic acid, and punicalagin in the effective fractions of PP. The HPLC analysis showed that ellagic acid, gallic acid, and punicalagin constituted 61.72% of PP, and they were determined to be the main active constituents of PP. Pomegranate was extensively referenced in medical folklore. Its various extracts contain a rich complement of steroidal and related polyphenolic compounds, some of which (e.g., luteolin, kaempferol, and quercetin) are estrogenic. Pomegranate pericarp is also very rich in tannins (gallic acid, ellagic acid), which, though not estrogenic, are strongly antioxidant. Both classes of compounds inhibit carcinogenesis and possess additional anticancer properties. The pomegranate fractions studied, though crude materials, showed a level of *in vivo* activity comparable to that of an equivalent dose of the pure isoflavone genistein (6).

In this study, PP inhibited cell proliferation and induced apoptosis in PC-3 cells. During normal proliferation of tumor cells, some of the cells undergo apoptosis. Subsequently, tumor necrosis will be triggered if apoptotic cells are not phagocytosed by macrophages within a short period of time. PP likely induces tumor cell necrosis and apoptosis by increasing TNF-α levels and reducing VEGF levels. PP could also prevent formation of new blood vessels in tumor cells, thus suppressing tumor growth and leading to necrosis. The antiproliferative and proapoptotic properties of pomegranate fruit extract (PFE) against human CaP cells have been demonstrated in cell culture systems and xenograft mouse models. Human PC-3 cells treated with PFE (10-100 μg/mL) for 48 h showed dose-dependent inhibition of cell growth and induction of apoptosis. The induction of apoptosis and cell cycle arrest by PFE was associated with up-regulation of proapoptotic Bax and Bak, down-regulation of anti-apoptotic Bcl-XL and Bcl-2, induction of WAF1/p21 and KIP1/p27, decreased levels of cyclins D1, D2, and E, and decreased protein expression of cyclin-dependent kinase (CDK)-2, CDK-4, and CDK-6 (3). Further studies are needed to elucidate the precise mechanism of action of PP in PC-3 cells. In the literature, the suggested mechanisms by which polyphenols produce anticancer effects (in addition to their role as potent antioxidants) include the following: 1) inhibition of human cancer cell growth by interfering with growth factor receptor signaling and cell cycle progression, 2) promotion of cellular

differentiation, 3) induction of hepatic xenobiotic enzyme activity that may provide additional defense mechanisms against oxidative stress and carcinogens, 4) inhibition of cholesterogenesis, 5) modulation of phosphodiesterase and cyclooxygenase pathways, 6) inhibition of protein kinases involved in cell signaling, and 7) inhibition of inflammation (8).

Numerous dietary agents have been investigated for their potential beneficial effects against CaP. The effects of pomegranate have been investigated in cell culture systems, animal models, and in a phase II clinical trial. PP showed supra-additive inhibitory effects on proliferation of human prostate cancer cells and induced human prostate cancer cell apoptosis. Moreover, PP increased serum levels of TNF-α and decreased serum levels of VEGF. Our results show that ellagic acid, gallic acid, and punicalagin are the main effectors of the anti-tumor activity of PP and offer empirical evidence of the anti-tumor effects of PP.

Acknowledgment This study was supported by Xinjiang Uighur science and technology plan projects (201233135).

Disclosure The authors declare no conflict of interest.

References

1. Sporn MB, Suh N. Chemoprevention of cancer. Carcinogenesis 21: 525-530 (2000)
2. Boone CW, Bacus JW, Bacus JV, Steele VE, Kelloff GJ. Properties of intraepithelial neoplasia relevant to the development of cancer chemopreventive agents. J. Cell Biochem. 67: 1-20 (1997)
3. Adhami VM, Ahmad N, Mukhtar H. Molecular targets for green tea in prostate cancer prevention. J. Nutr. 133: 2417S-2424S (2003)
4. Siddiqui IA, Adhami VM, Saleem M, Mukhtar H. Beneficial effects of tea and its polyphenols against prostate cancer. Mol. Nutr. Food Res. 50: 130-143 (2006)
5. Lansky EP, Newman RA. *Punica granatum* (pomegranate) and its potential for prevention and treatment of inflammation and cancer. J. Ethnopharmacol. 109: 177-206 (2007)
6. Albrecht M, Jiang W, Kumi-Diaka J, Lansky EP, Gommersall LM, Patel A, Mansel RE, Neeman I, Geldof AA, Campbell MJ. Pomegranate extracts potently suppress proliferation, xenograft growth, and invasion of human prostate cancer cells. J. Med. Food 7: 274-283 (2004)
7. Kim ND, Mehta R, Yu WP, Neeman I, Livney T, Amichay A, Poirier D, Nicholls P, Kirby A, Jiang W, Mansel R, Ramachandran C, Rabi T, Kaplan B, Lansky E. Chemo preventive and adjuvant therapeutic potential of pomegranate (*Punica granatum*) for human breast cancer. Breast Cancer Res. Treat. 71: 203-217 (2002)
8. Allan J, Pantuck MD, Nazy Zomorodian MD, Arie S, Belldegrun MD. Phase-II study of pomegranate juice for men with prostate cancer and increasing PSA. In: American Urological Association Annual Meeting, May 22-25, San Antonio, TX, USA (2005)
9. Ephraim PL, Gregory H, Paul F, Wen G. Jiang. Pomegranate (*Punica granatum*) pure chemicals show possible synergistic inhibition of human PC-3 prostate cancer cell invasion across Matrigel^{TM}. Invest. New Drug. 23: 121-122 (2005)
10. Negi PS, Jayaprakasha GK, Jena BS. Antioxidant and anti-mutagenic activities of pomegranate peel extracts. Food Chem. 80: 393-397 (2003)
11. Adhami VM, Khan N, Mukhtar H. Cancer chemoprevention by pomegranate: Laboratory and clinical evidence. Nutr. Cancer 61: 811-815 (2009)
12. Zhang QQ, Long LM, Wang WJ, Yang SL, Tang LH, Liang ZQ. The inhibition activity of the active extraction *Lysimachia clethroides* Duby on Hela xenograft in nude mice. Chinese Pharmacol. Bull. 28: 204-208 (2012)
13. Wang DY, Zhang HQ, Li X. Apoptosis induced by the C21 sterols in Baishouwu and its mechanism of action in hepatoma. Acta Pharm. Sinic. 42: 366-370 (2007)
14. Li L, Xu LJ, Peng Y, He ZD, Shi RB, Xiao PG. Simultaneous determination of five phenylethanoid glycosides in small-leaved Kudingcha from the *Ligustrum* genus by UPLC/PDA. Food Chem. 131: 1583-1588 (2012)
15. Wang QX, Jia MY, Tang RP, Xu L,Yang YH. A review of chemical constituent and application of *Punica granatum* Seed. Spec. Wild Econ. Anim. Plant Res. 1: 53-56 (2006)
16. Pitchakarn P, Chewonarin T, Ogawa K, Suzuki S, Asamoto M, Takahashi S, Shirai T, Limtrakul P. Ellagic acid inhibits migration and invasion by prostate cancer cell lines. Asian Pac. J. Cancer P. 14: 2859-2863 (2013)
17. Naiki-Ito A, Chewonarin T, Tang M, Pitchakarn P, Kuno T, Ogawa K, Asamoto M, Shirai T, Takahashi S. Ellagic acid, a component of pomegranate fruit juice, suppresses androgen-dependent prostate carcinogenesis via induction of apoptosis. Prostate 75: 151-160 (2015)
18. Wang L, Li W, Lin M, Garcia M, Mulholland D, Lilly M, Martins-Green M. Luteolin, ellagic acid and punicic acid are natural products that inhibit prostate cancer metastasis. Carcinogenesis 35: 2321-2330 (2014)