JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY
ARTICLE

J. Agric. Food Chem. 2010, 58, 12149–12156 12149
DOI:10.1021/jf103306k

Growth Inhibitory, Antiandrogenic, and Pro-apoptotic Effects of Punicic Acid in LNCaP Human Prostate Cancer Cells

Jihane Gasmi and J. Thomas Sanderson*

Institut National de la Recherche Scientifique, Institut Armand-Frappier, Université du Québec, Laval, Québec H7 V1B7, Canada

Prostate cancer is a commonly diagnosed cancer in men, and dietary chemoprevention by pomegranate (*Punica granatum*) extracts has shown noticeable benefits. In this study, we investigated the growth inhibitory, antiandrogenic, and pro-apoptotic effects of 13 pure compounds found in the pomegranate in androgen-dependent LNCaP human prostate cancer cells. Cells deprived of steroid hormones were exposed to increasing concentrations (1−100 μM) of pomegranate compounds in the presence of 0.1 nM dihydrotestosterone (DHT), and the inhibition of cell growth was measured by WST-1 colorimetric assay after a 4 day exposure. Four compounds, epigallocatechin gallate (EGCG), delphinidin chloride, kaempferol, and punicic acid, were found to inhibit DHT-stimulated cell growth at concentrations of 10 μM and above. These four pomegranate compounds inhibited DHT-stimulated androgen receptor nuclear accumulation and the expression of the androgen receptor-dependent genes prostate specific antigen and steroid 5α-reductase type 1 at concentrations ≥10 μM. We determined the possible contribution of apoptosis to the observed decrease in cell growth and found that three compounds, EGCG, kaempferol, and, in particular, punicic acid, induced DNA fragmentation after a 24 h treatment, at concentrations in the 10−100 μM range. Punicic acid, an important fatty acid in pomegranate seeds, was further found to induce intrinsic apoptosis via a caspase-dependent pathway. In conclusion, punicic acid, the main constituent of pomegranate seed (70−80%), exhibited potent growth inhibitory activities in androgen-dependent LNCaP cells, which appear to be mediated by both antiandrogenic and pro-apoptotic mechanisms.

KEYWORDS: Pomegranate; punicic acid; SRD5A1; PSA; LNCaP; apoptosis

1. INTRODUCTION

Prostate cancer is the most common cancer diagnosed in North American men and is the third leading cause of cancer death in western countries (*1*). The number of cases diagnosed has increased over the last decades in part due to the development of sensitive tests for the detection of prostate cancer markers, such as prostate-specific antigen (PSA), which are expressed androgen dependently and are secreted into the blood circulation (*2*).

Most prostate cancers are initially androgen-dependent, and the main treatment option at this early stage is androgen ablation (*3*,*4*). However, after an initial successful response to androgen ablation therapy, most prostate cancers progress to an androgen-resistant state that is highly aggressive, metastatic, and often leads to death (*5*).

Compelling risk factors involved in the development and progression of prostate cancer include older age, a family history of prostate cancer, and race. Increasing interest has centered on nutritional or other environmental factors that either offer protection against prostate cancer or increase its incidence. Asian men have much lower incidences of prostate cancer and benign prostatic hyperplasia (BPH) than their Western counterparts (*6*). Moreover, vegetarian men have a lower incidence of prostate cancer than omnivorous males. Taken together, both populations of men consume low-fat, high-fiber diets containing fruits and vegetables, which provide a rich supply of antioxidants, weak dietary estrogens, and possible antiandrogens. This implies that environmental factors, rather than genetics alone, contribute substantially to the risk of developing prostate cancer (*7*).

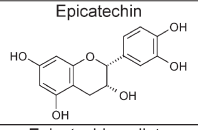
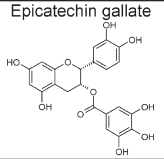
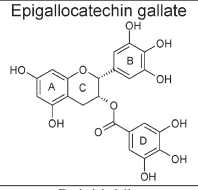
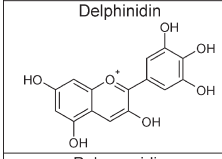
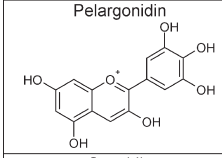
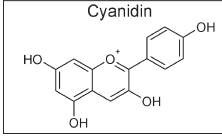
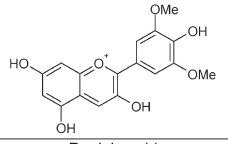
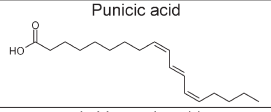
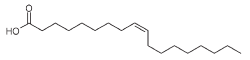
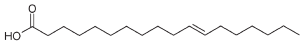
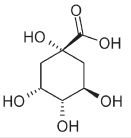
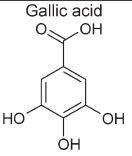
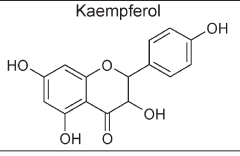
Dietary chemoprevention of prostate cancer is increasingly considered to be an important way to reduce this health problem (*8*,*9*). Recently, pomegranate (*Punica granatum*), a source of many polyphenols including ellagitannins, punicaligins, gallotannins, anthocyanins, and flavonoids, has shown promise in the prevention of prostate cancer, as has been demonstrated in studies with extracts and/or isolated bioactive compounds (*10*−*12*). More recently, ellagitannin metabolites formed in the gut, such as urolithin A and B, are being considered as biologically active agents against prostate cancer (*13*); they appear to have protective (anti-inflammatory) actions in colon (*14*), although little is yet known about their concentrations in prostate, therapeutically relevant levels, or mechanism of action.

We hypothesize that certain compounds found in the pomegranate have growth inhibitory and antiandrogenic properties that contribute to their protection against the development and growth of prostate cancer. The objective of the present study was

*To whom correspondence should be addressed. Tel: 450-687-5010 ext. 8819. Fax: 450-686-5309. E-mail: thomas.sanderson@iaf.inrs.ca.

Published on Web 11/10/2010

Table 1. Thirteen Pomegranate Compounds Used in This Study, Their Predominant Localization in the Fruit, and Approximate Concentrations

Pomegranate compound	Approximate concentrations	Source (*12*)	Reference
Epicatechin 	0.8 mg/L	Juice with peel	(*49*)
Epicatechin gallate 	1.6 mg/L	Juice with peel	(*49*)
Epigallocatechin gallate 	Quantitative data not available	Juice with peel, leaf	
Delphinidin 	237.8 mg/L (as glucosides)	Juice with peel	(*47*)
Pelargonidin 	5.9 mg/L (as glucosides)	Juice with peel	(*47*)
Cyanidin 	181.3 mg/L (as glucosides)	Juice with peel	(*47*)
Malvinidin 	Quantitative data not available	Juice with peel	
Punicic acid 	67-80% of all fatty acids	Seed oil	(*50*)
cis-Vaccenic acid 	Quantitative data not available	Seed oil	
trans-Vaccenic acid 	Quantitative data not available	Seed oil	
Quinic acid 	0.02–6.72 g/L	Juice with peel	(*51*)
Gallic acid 	Quantitative data not available	Juice, peel flower	
Kaempferol 	Quantitative data not available	Juice, leaf	

to examine the potential protective effects of a number of pure compounds known to be found in various parts of the pomegranate, including punicic acid, which is the predominant trienoic acid in this fruit (*15*). The effects of pomegranate compounds on cell growth and apoptosis were examined in an androgen-dependent and androgen receptor (AR)-positive human prostate cancer cell line (LNCaP). Because androgens and the AR play central roles throughout prostate cancer development, the effects of pomegranate polyphenols and fatty acids were evaluated on the transcription of genes for the AR-dependent dihydrotestosterone (DHT)-synthesizing enzyme SRD5A1 (steroid 5 reductase type 1), PSA, and nuclear AR levels.

2. MATERIALS AND METHODS

2.1. Pomegranate Compounds. Punicic acid, gallic acid, *cis*-vaccenic acid, *trans*-vaccenic acid, and quinic acid were purchased from Larodan Fine Chemicals AB (Malmö, Sweden), and kaempferol, delphinidin chloride, pelargonidin chloride, cyanidin chloride, malvidin chloride, epicatechin (EC), epicatechin gallate (ECG), and epigallocatechin gallate (EGCG) were purchased from Extrasynthèse (Genay, France) (**Table 1**). All compounds were dissolved in dimethyl sulfoxide (DMSO) as 1000-fold concentrated stock solutions. Although some compounds are water-soluble, we chose DMSO for all solutions to remain consistent. The quantitative pomegranate contents of these compounds in the actual fruit, as far as known, are summarized in **Table 1**.

2.2. Cell Culture. Androgen-dependent LNCaP cells (American Type Culture Collection, Manassas, United States) were maintained and cultured in RPMI 1640 medium supplemented with 10% FBS and 1% penicillin/streptomycin in a humidified atmosphere of 5% CO_2 at 37 °C. Cells in 75 cm^2 flasks were grown to 80% confluence and then seeded in multiwell cell culture plates for the experimental procedures. Fresh batches of LNCaP cells were cryogenically frozen (in steps of 1 °C until −80 °C; then frozen in liquid nitrogen) at passage numbers 4−5 for future use. Passage numbers between 2 and 25 were used; after 25 passages, androgen responsiveness declined.

2.3. Cell Growth Inhibition Assay. LNCaP cells were seeded in 96-well plates (Fisher, Ottawa, ON) at a concentration of 5×10^4 cells/well in 100 μL of phenol red-free culture medium with 10% CS-FBS (charcoal-stripped fetal bovine serum) for 24 h, to arrest cell growth by removing steroids. Cells were then exposed to solvent (negative) control (0.1% DMSO), DHT (0.1 nM; positive control for stimulation of cell growth), or the pomegranate compounds (0, 1, 3, 10, 30 and 100 μM) in the presence of 0.1 nM DHT (an approximate EC_{50} for increased cell growth) for 48 h. The medium was then refreshed, and the cells were exposed for a further 48 h to the same experimental treatments. A WST-1 cell viability assay (Roche Diagnostics, Laval, QC) was performed according to the manufacturer's instruction. The absorbance was measured at 440 nm using a Spectramax M5 multifunctional spectrometer (Molecular Devices, Sunnyvale, CA).

2.4. Assay of Apoptosis. Cells were seeded at a concentration of 0.5×10^4 cells/well in 100 μL of complete culture medium in 96-well plates for 24 h. The pomegranate compounds (0, 10, 30 and 100 μM) were added, and the cells were incubated for an additional 24 h. A Cell Death Detection $ELISA^{PLUS}$ kit (Roche Diagnostics) was used to determine the formation of cytoplasmic histone-associated DNA fragments following the manufacturer's instructions.

2.5. Protein Isolation and Immunoblotting. A Ne-Per Nuclear and Cytoplasmic Extraction Reagent kit (Pierce Biotechnologies, Rockford, IL) was used for the extraction of nuclear and cytoplasmic protein fractions. Cell pellets were lysed in RIPA buffer containing 1X protease inhibitor cocktail, and protein concentrations were determined using the

Bradford assay. Proteins (15 μg) were resolved by sodium dodecyl sulfate−polyacrylamide gel electrophoresis and transferred to PVDF membrane (Bio-Rad, Mississauga, ON). Membranes were blocked with 5% reconstituted milk powder (Sobeys, Mississauga, ON) and then probed with the appropriate primary and secondary antibodies at 4 °C overnight. Antibody−antigen complexes were visualized by Immobilon Western Chemiluminescence reagents (Millipore, Billerica, MA) using a Versadoc imaging system (Bio-Rad). β-Actin and GAPDH were used as protein loading (reference) controls. The mouse antibodies raised against human β-actin, AR, and caspase-8 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The rabbit antibodies raised against human caspase-9, cleaved poly(ADP-ribose) polymerase (PARP), Bcl2, Bax, GSK-3β, and phospho-GSK-3β were purchased from Cell Signaling Technology (Danvers, MA). GAPDH, phospho-Akt1, and Akt1 antibodies were purchased from Millipore. Electrophoresis and immunoblotting equipment was purchased from Bio-Rad. LY294002 (Sigma-Adrich, St. Louis, MO), a phosphatidylinositol-3-kinase (PI3K) inhibitor, was used as a positive control for inhibition of Akt phosphorylation at the Ser473 position.

2.6. mRNA Extraction and Reverse Transcription Polymerase Chain Reaction (RT-PCR). LNCaP cells were seeded at a concentration of 3×10^5 cells/well in 2 mL of phenol red-free with CS-FBS in 6-well plates for 24 h to arrest cell growth. Cells were then exposed for 24 h to solvent (negative) control (0.1% DMSO), DHT (0.1 nM; an approximate EC_{50} value for PSA induction and positive control for stimulation of AR-dependent gene expression), or the pomegranate compounds (0, 3, 10, 30, 100 μM) in the presence of 0.1 nM DHT. Total RNA was prepared using a High Pure RNA Isolation Kit (Roche Diagnostics) and was reverse transcribed and amplified using a one-step Access RT-PCR kit (Promega, Madison, WI). RT-PCR reactions were performed in 25 μL containing 100 ng of RNA and 23 μL of MasterMix. Oligonucleotide primers (Invitrogen, Carlsbad, CA) were included in the reactions with sequences as follows: PSA (accession number M26663, NCBI GenBank): 5′-GCC TCT CGT GGC AGG GCA GT-3′ (forward), 5′-CTG AGG GTG AAC TTG CGC AC-3′ (reverse); SRD5A1 (accession number M32313): 5′-GCG AGG AGG AAA GCC TAT GC-3′ (forward), 5′-CAG GGC ATA GCC ACA CCA CT-3′ (reverse); β-actin (accession number M10277): 5′-GTA CCC TGG CAT TGC CGA C-3′ (forward), 5′-TAA CGC AAC TAA GTC ATA GTC C-3′ (reverse). Cycling conditions for PSA were as follows: denaturing at 95 °C for 1 min, annealing at 58 °C for 1 min, extension at 72 °C for 2 min for 35 cycles. Cycling conditions for SRD5A1 and β-actin were as follows: denaturing at 95 °C for 1 min, annealing at 59 °C for 1 min, extension at 72 °C for 2 min for 35 cycles. These conditions were chosen to produce amplified products in the exponential phase, assuring that semiquantitative results were obtained.

2.7. Statistical Analyses. All treatments were performed in quadruplicate (growth inhibition experiments) or in triplicate (immunoblotting and apoptosis experiments) per experiment, and each experiment was performed three times independently. All densitometric analyses of immunoblots were expressed as normalized means ± standard deviations (SDs). Statistically significant differences ($*p < 0.05$) were determined using one-way analysis of variance (ANOVA) with Tukey posterior test. IC_{50} values were calculated using nonlinear curve−fit analysis and are presented as means ±95% confidence intervals, based on the average of three independent experiments. All analyses were performed using GraphPad Prism v5.03 (GraphPad Software, San Diego, CA).

3. RESULTS

3.1. Growth Inhibitory and Antiandrogenic Effects of Pomegranate Compounds. LNCaP cells, after initial deprivation of steroids (24 h), were exposed to 13 pomegranate compounds for 48 h, in the presence of a growth-inducing (0.1 nM) concentration of DHT. DHT (0.1 nM) alone stimulated cell growth by 3−4-fold, and control cells increased in number by about 2% (not shown). Only four pomegranate compounds displayed consistent concentration-dependent (3−100 μM) growth inhibition of DHT-stimulated LNCaP cells. These were EGCG, delphinidin chloride, kaempferol, and punicic acid (**Figure 1**), with IC_{50} values of 34 ± 8, 38 ± 7, 29 ± 6, and 18 ± 4 μM, respectively. LNCaP

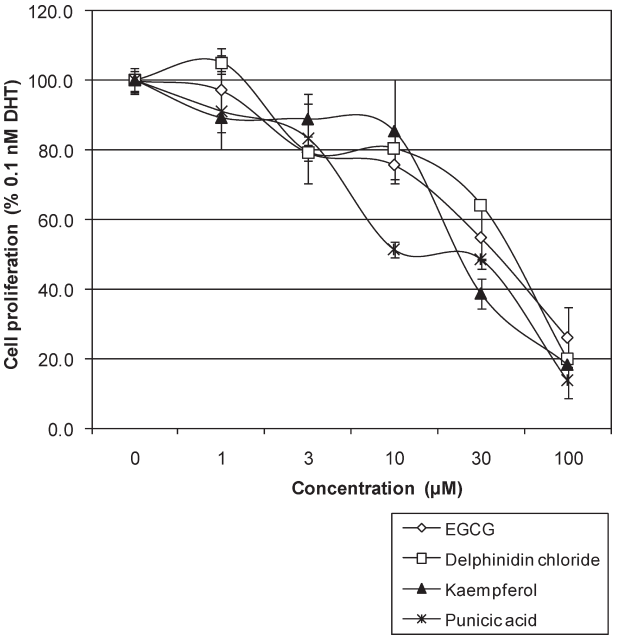


Figure 1. Effects of EGCG, delphinidin chloride, kaempferol, and punicic acid on growth and survival of LNCaP cells. Cells were cultured with increasing concentrations of pomegranate compounds for 4 days (with a redosing after 48 h, see section 2.3) in phenol red- and steroid-free medium in the presence of 0.1 nM DHT, and cell viability was determined by WST-1 assay. Negative control cells received 1 μL of DMSO (and demonstrated negligible cell growth; about 2% of that of 0.1 nM DHT), and positive control cells were exposed to 1 μL of DHT (0.1 nM) to stimulate cell growth. The average ± SD of three independent experiments is shown. IC_{50} values were calculated as described in section 2.7 and are listed in section 3.1.

cells, conditioned in the same way as in the growth inhibition experiments, were incubated with increasing concentrations (3−100 μM) of EGCG, delphinidin chloride, kaempferol, or punicic acid in the presence of 10 nM DHT (the DHT concentration required to produce a clear increase in AR protein in nucleus) for 24 h. DHT-induced AR nuclear accumulation (determined by immunoblotting) was inhibited by EGCG at concentrations ≥10 μM (**Figure 2A**) and by delphinidin chloride, kaempferol, and punicic acid at a concentration of 100 μM (**Figure 2B−D**). None of the pomegranate compounds were cytotoxic when tested in the absence of DHT (not shown). In a parallel experiment using the same conditions, total RNA was extracted, and levels of PSA, SRD5A1, and β-actin mRNA were determined by semiquantitative RT-PCR. DHT-stimulated expression of PSA and SRD5A1 was inhibited at concentrations ≥3 μM for punicic acid (**Figure 3D**,**H**) and ≥30 μM for EGCG (**Figure 3A**,**E**) and kaempferol (**Figure 3C**, **G**) and at 100 μM for delphinidin chloride (**Figure 3B**) but without a statistically significant effect on the expression of SRD5A1 (**Figure 3F**).

3.2. Do Growth Inhibitory Pomegranate Compounds Induce Apoptosis in LNCaP Cells? To test whether the decrease in cell growth by these compounds was due to induction of apoptosis, we determined LNCaP nuclear DNA fragmentation after a 24 h exposure to the four pomegranate compounds (10, 30, and 100 μM) in complete culture medium. EGCG and kaempferol induced apoptosis above 30 μM in LNCaP cells. However, punicic acid was the most potent compound since it induced apoptosis at ≥10 μM (**Figure 4**) and was selected for further experiments.

Cells were treated with increasing concentrations of punicic acid (0, 3, 10, 30, and 100 μM) in complete culture medium for 24 h. A reduction in Bcl2 expression by punicic acid was seen at 30 and

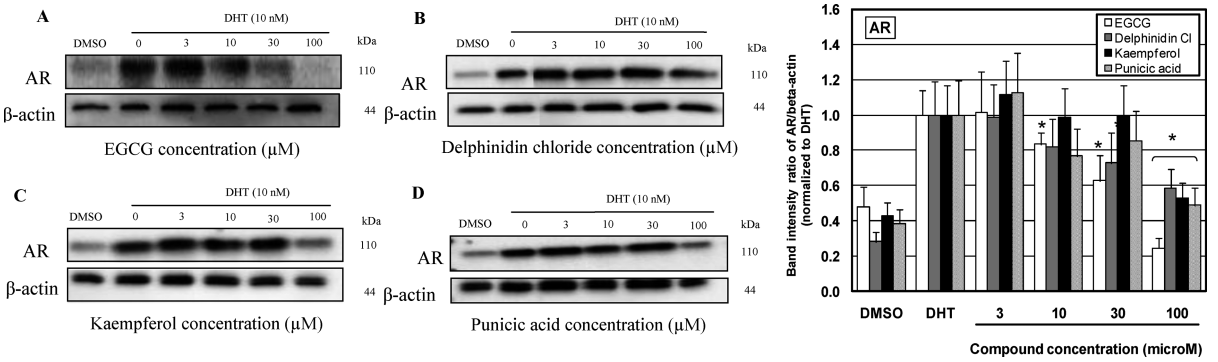


Figure 2. Nuclear levels of AR protein. LNCaP cells were incubated with increasing concentrations of (**A**) EGCG, (**B**) delphinidin chloride, (**C**) kaempferol, or (**D**) punicic acid in phenol-red and steroid-free medium for 24 h in the presence of 10 nM DHT. Protein levels of AR in the nucleus were determined by immunoblot analysis. Equal loading of proteins was verified by stripping and reprobing the blots with β-actin antibodies. Negative control cells received 0.1% DMSO, and positive control cells were exposed to 10 nM DHT in 0.1% DMSO to induce nuclear accumulation of AR. All treatments were normalized to the DHT control. The densitometric analyses to the right represent the mean $\pm$ SD of three independent experiments, where an * indicates a statistically significant decrease in nuclear AR levels (one-way ANOVA, $p < 0.05$). An * above a bracket indicates that all four treatments were significantly different from the DHT control.

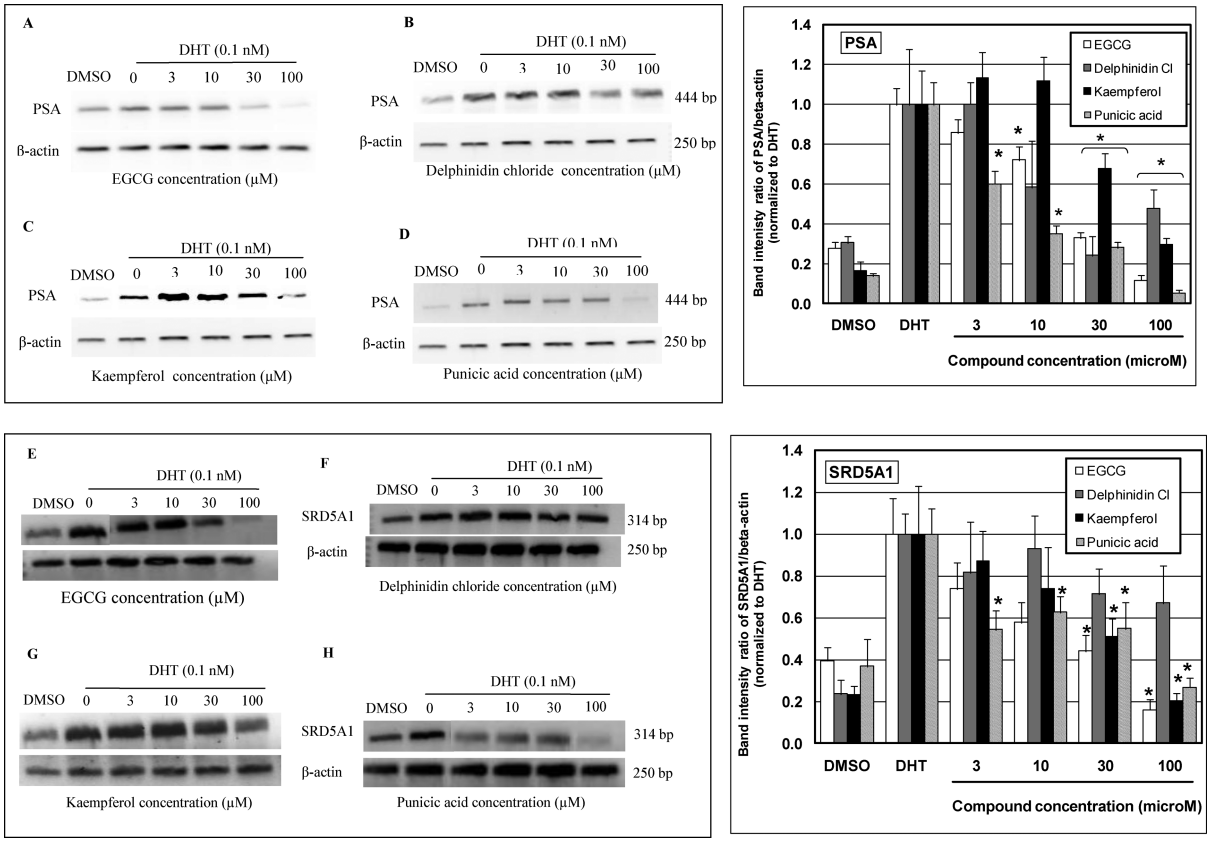


Figure 3. RT-PCR analysis of the expression of androgen-dependent prostate marker genes (PSA and SRD5A1, respectively) in LNCaP cells treated with increasing concentrations of EGCG (**A**, **E**), delphinidin chloride (**B**, **F**), kaempferol (**C**, **G**), or punicic acid (**D**, **H**) in phenol red- and steroid-free medium in the presence of DHT (0.1 nM) for 24 h. Total RNA was extracted, and levels of PSA and β-actin mRNA were determined by RT-PCR. Negative control cells received 0.1% DMSO, and positive control cells were exposed to 10 nM DHT in 0.1% DMSO to induce nuclear accumulation of AR. All treatments were normalized to the DHT control. The densitometric analyses to the right each represent the mean $\pm$ SD of three independent experiments, where an * indicates a statistically significant decrease in either PSA or SRD5A1 expression levels (one-way ANOVA, $p < 0.05$). An * above a bracket indicates that all four treatments were significantly different from the DHT control.

100 μM, indicating that disintegration of mitochondrial structures had occurred, resulting in initiation of the apoptotic pathway, likely via activation of the caspase 9 cascade as we detected caspase 9 cleavage (**Figure 5**). Further evidence in favor of apoptosis was chromatin cleavage resulting in oligonucleosomal DNA fragmentation and cleavage of PARP. PARP is one of the targets of active caspase-3, and punicic acid induced its cleavage into an 89 kDa fragment after 24 h of exposure, which was statistically significant at a concentration $\geq$30 μM. Punicic acid further caused inactivation of Akt via reduction of the active phospho-Akt-Ser 473 and reduced inactivation of GSK-3β by decreasing the inactive phospho-GSK-3β-Ser 9 at $\geq$30 μM, after 1 h of exposure (**Figure 6**).

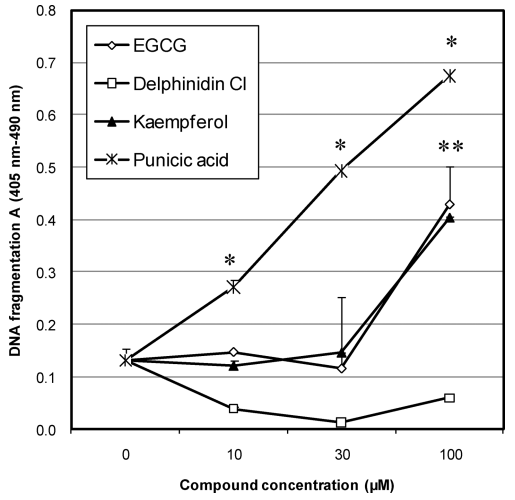


Figure 4. Apoptotic effects of EGCG, delphinidin chloride, kaempferol, and punicic acid in LNCaP cells. Cells in complete culture medium were exposed to the four compounds for 24 h. Apoptosis was determined by a Cell Death Detection ELISA^{PLUS} kit. The combination of three independent experiences is shown. An * indicates statistically significant differences from vehicle control (one-way ANOVA, $p < 0.05$).

4. DISCUSSION

Prostate cancer is an important malignant disease in men, particularly in western countries. The number of new cases in United States in 2009 was estimated to be almost 200000 with an estimated death toll of almost 30000 (*16*). To prevent and control this disease more effectively, there is a need to identify chemopreventive approaches (*11*, *17*−*19*). Pomegranate fruits, juices, and extracts have been used extensively in ancient cultures for various medicinal purposes (*20*). Pomegranate polyphenols are potent antioxidants that have been shown both in vitro and in vivo to inhibit the growth of prostate and certain other forms of cancer (*21*−*23*).

In this study, we have demonstrated that 4 out of the 13 pomegranate compounds that we tested (**Table 1**), EGCG, delphinidin chloride, kaempferol, and punicic acid, had growth inhibitory effects (**Figure 1**) and pro-apoptotic activities (delphinidin chloride excepted) (**Figure 4**) in LNCaP androgen-dependent human prostate cancer cells. All four compounds significantly inhibited the expression levels of AR proteins in a concentration-dependent manner (**Figure 2**), as well as reducing the DHT-mediated upregulation of PSA and steroid 5α-reductase SRD5A1 gene expression in LNCaP cells (**Figure 3**). Inhibition of gene expression of androgen synthesizing enzymes and reduction of nuclear AR levels may contribute to the growth inhibitory effects of these compounds.

LNCaP cells express Ar and their growth is androgen-dependent. Cell growth requires AR activation and local steroid 5α-reduction of circulating testosterone to produce the potent androgen DHT (*24*), which is an effective inducer of transcription of PSA, resulting in increased synthesis and secretion of PSA protein. Our study shows that four pomegranate compounds were able to down-regulate the expression of these AR-dependent marker genes in LNCaP cells (**Figure 3**), as well as reduce nuclear AR levels (**Figure 2**), indicating that these compounds not only interfere with AR-dependent cell growth but also affect the conversion of testosterone into the more potent DHT. In the case of EGCG, our findings confirm studies that have shown that catechins (EGCG) present in green tea, polyunsatured fatty acids such as γ-linolenic acid, ellagitannins, the flavonoids, and lignans, are also inhibitors of steroid 5α-reductase (*25*−*27*). Although the structures of EGCG and EG are very similar, only EGCG had significant antiandrogenic effects. This is likely due to the additional electron donating hydroxy group on the phenyl B ring of EGCG, which increases the electronegative properties of this phenolic moiety. It is known that the interaction of DHT with the ligand-binding pocket of the AR is driven predominantly by electrostatic bonds (*28*, *29*). Thus, the highly hydroxylated EGCG has the greatest potential to interfere with the binding of DHT to the AR. In support of this, electron spin resonance studies have shown that EGCG is considerably more electrostatically charged than its EC analogues that lack having three hydroxygroups on the B ring (*30*). This study also showed that the gallate group (the hydroxylated D ring; **Table 1**) had no influence.

The present study has demonstrated that out of the four most growth inhibitory and antiandrogenic pomegranate compounds selected, punicic acid was the most potent inducer of apoptosis in LNCaP cells (**Figure 4**). It has previously been shown to inhibit breast cancer proliferation and induce apoptosis in MDA-MB-231 and MDA-ERα7 cells (*31*). Several apoptosis-associated genes or proteins have been shown to play critical roles in regulating apoptosis. These include caspases, Bcl2 family members, and PARP (*32*, *33*). To determine whether these proteins are involved in the mediation of punicic acid-induced inhibition of LNCaP cell growth, we examined caspase activation and cleavage of PARP by Western blotting (**Figure 5**). Bcl2 is an upstream effector molecule in the intrinsic apoptotic pathway and has been identified as a potent suppressor of apoptosis (*12*). Most cancers, including prostate cancer, generally overexpress Bcl2 (*34*, *35*) thereby escaping apoptosis and undermining effective therapy. Bcl2 forms a heterodimer with the apoptotic protein Bax and neutralizes its apoptotic effects. We showed that punicic acid, the most abundant trienoic fatty acid found in the pomegranate, significantly decreased Bcl2 protein levels after a 24 h of treatment without affecting the level of Bax protein (**Figure 5**). These results suggest that punicic acid-induced apoptosis is mediated via down-regulation of Bcl2 antiapoptotic proteins together with a decreased Bcl2/Bax ratio in LNCaP cells. Alteration of this ratio is a decisive factor in whether cells will undergo apoptosis. Down-regulation of Bcl2 results in cytochrome *c* release from the mitochondria, initiating an apoptotic process via activation of caspase 9. Activated caspase 9 cleaves procaspase 3 to form activated caspase 3, which in turn cleaves poly(ADP-ribose) polymerase (PARP) , allowing DNA degradation to continue. Taken together, these data clearly indicate that punicic acid is an efficient inducer of apoptosis via this pathway in LNCaP cells.

Apoptosis is a normal cellular function that controls excessive proliferation by eliminating unnecessary or damaged cells. Cancer cells have devised several mechanisms to inhibit apoptosis and prolong their survival. Akt serves as one of the major antiapoptotic factors in apoptotic pathway. Akt is constitutively activated in LNCaP cells (*36*); therefore, it is possible that Akt provides survival support to prostate cancer cells in the absence of androgens. Because we have shown that punicic acid inhibits the phosphorylation of Akt, survival support of these cells is eliminated, and they are driven toward apoptosis (**Figure 6**). The principal characterized physiological substrate of Akt is glycogen synthase kinase-3β (GSK-3β) (*37*), which was initially identified as an enzyme that regulates glycogen synthesis in response to insulin (*38*). GSK-3β is an ubiquitously expressed protein-serine/threonine kinase whose activity is inhibited by Akt phosphorylation in response to growth factor stimulation. Here, we show that GSK-3β is involved in the regulation of apoptosis, identifying it as a critical downstream element of the PI 3-kinase/Akt cell survival pathway (**Figure 6**), although identification of the GSK-3β targets that regulate apoptosis is a critical next step. Also, our

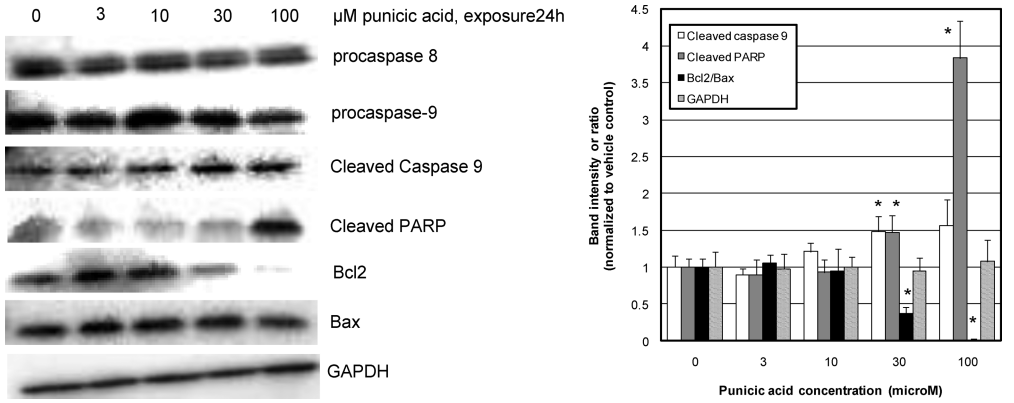


Figure 5. Punicic acid activates caspase-9 and mitochondrial apoptosis in prostate cancer cells. Concentration-dependent caspase-9 processing and cleavage of the caspase-3 substrate PARP after 24 h of treatment in LNCaP cells. One of three experiments is shown. The densitometric analyses to the right reflect the normalized average band intensities of all three experiments. An * indicates significant differences from DMSO control (one-way ANOVA, $p < 0.05$).

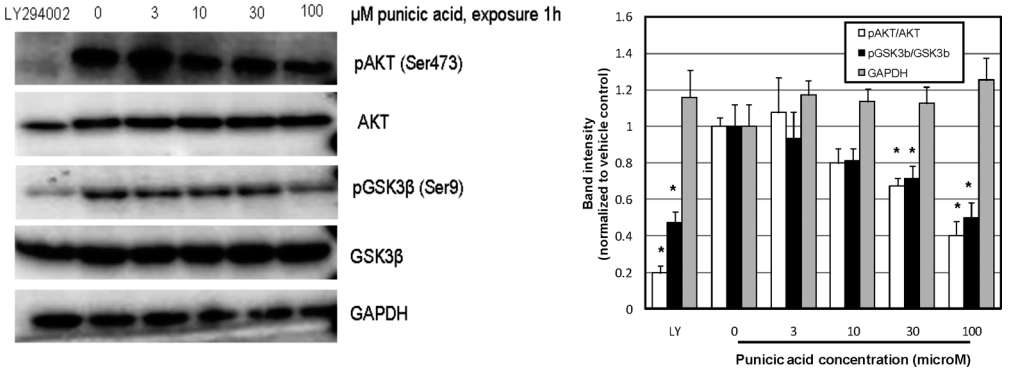


Figure 6. Punicic acid decreased phosphorylation of Akt at Ser473 (active form) above 10 μM and that of GSK-3β at Ser9 (inactive form) at 100 μM in LNCaP cells after a 1 h of exposure. The PI-3-kinase inhibitor LY294002 (30 μM) was used as a positive control. One of three experiments is shown. The densitometric analyses reflect the normalized average band intensities of all three experiments. An * indicates significant differences from DMSO control (one-way ANOVA, $p < 0.05$).

observation that both Akt and caspase 9 are associated with apoptosis induced by punicic acid brings up the question whether both pathways are activated independently or that they interact.

Our findings confirm studies that have shown that EGCG, delphinidin chloride, and kaempferol have antitumor effects on the prostate cancer cell line LNCaP (*18*, *39*−*46*). However, we are the first to show that punicic acid, with predominant presence in pomegranate seed oil, has greater pro-apoptotic effects than EGCG, kaempferol, or delphinidin chloride. We postulate that mixtures of the numerous biologically active compounds in the pomegranate may have additive or synergistic effects resulting in more effective inhibition of cell growth than any one single compound. As these compounds appear to have different/complementary mechanisms of growth inhibitory action, they could also be effective agents for the treatment of both androgen-dependent and androgen-independent prostate cancer cells (*47*, *48*). We will address the above hypotheses in the future. Future studies should furthermore focus on the bioavailable metabolites that are formed and their biologically relevant concentrations in plasma and ideally in target organs of interest such as the prostate.

In conclusion, our results show that among several other compounds found in the pomegranate, punicic acid has a relatively potent growth inhibitory and antiandrogenic effect on androgen-dependent LNCaP cells. This is also the first study to implicate Akt as a key mediator of apoptotis induced by punicic acid. Impairment of the PI3K/Akt pathway by punicic acid appears to be an important and relevant chemotherapeutic mechanism against prostate cancer growth.

ABBREVIATIONS USED

AR, androgen receptor; CS-FBS, charcoal stripped fetal bovine serum; DMSO, dimethyl sulfoxide; DHT, dihydrotestosterone; PSA, prostate-specific antigen; SRD5A1, steroid 5α-reductase type 1.

ACKNOWLEDGMENT

We thank Dave Lanoix, Patricia Rivest, and Talal Oufkir for their technical assistance with the experiments. We also thank Dr. Ephraim Lansky for his important advice.

LITERATURE CITED

(1) Heidenreich, A.; Aus, G.; Bolla, M.; Joniau, S.; Matveev, V. B.; Schmid, H. P.; Zattoni, F. EAU guidelines on prostate cancer. *Eur. Urol.* **2008**, *53* (1), 68−80.

(2) Ulmert, D.; Cronin, A. M.; Bjork, T.; O'Brien, M. F.; Scardino, P. T.; Eastham, J. A.; Becker, C.; Berglund, G.; Vickers, A. J.; Lilja, H. Prostate-specific antigen at or before age 50 as a predictor of advanced prostate cancer diagnosed up to 25 years later: A case-control study. *BMC Med.* **2008**, *6*, 6.

(3) Aquilina, J. W.; Lipsky, J. J.; Bostwick, D. G. Androgen deprivation as a strategy for prostate cancer chemoprevention. *J. Natl. Cancer Inst.* **1997**, *89* (10), 689−696.

(4) Cleutjens, K. B.; van Eekelen, C. C.; van der Korput, H. A.; Brinkmann, A. O.; Trapman, J. Two androgen response regions cooperate in steroid hormone regulated activity of the prostate-specific antigen promoter *J. Biol. Chem.* **1996**, *271* (11), 6379−6388.

(5) Feldman, B. J.; Feldman, D. The development of androgen-independent prostate cancer. *Nat. Rev. Cancer* **2001**, *1* (1), 34−45.

(6) Shimizu, H.; Ross, R. K.; Bernstein, L.; Yatani, R.; Henderson, B. E.; Mack, T. M. Cancers of the prostate and breast among Japanese and white immigrants in Los Angeles County. *Br. J. Cancer* **1991**, *63* (6), 963−966.
(7) Fleshner, N.; Zlotta, A. R. Prostate cancer prevention: past, present, and future. *Cancer* **2007**, *110* (9), 1889−1899.
(8) Moorthy, H. K.; Venugopal, P. Strategies for prostate cancer prevention: Review of the literature. *Indian J. Urol.* **2008**, *24* (3), 295−302.
(9) Singh, R. P.; Agarwal, R. Mechanisms of action of novel agents for prostate cancer chemoprevention. *Endocr.-Relat. Cancer* **2006**, *13* (3), 751−778.
(10) Trottier, G.; Bostrom, P. J.; Lawrentschuk, N.; Fleshner, N. E. Nutraceuticals and prostate cancer prevention: A current review. *Nat. Rev. Urol.* **2010**, *7* (1), 21−30.
(11) Syed, D. N.; Suh, Y.; Afaq, F.; Mukhtar, H. Dietary agents for chemoprevention of prostate cancer. *Cancer Lett.* **2008**, *265* (2), 167−176.
(12) Lansky, E. P.; Newman, R. A. *Punica granatum* (pomegranate) and its potential for prevention and treatment of inflammation and cancer. *J. Ethnopharmacol.* **2007**, *109* (2), 177−206.
(13) Gonzalez-Sarrias, A.; Gimenez-Bastida, J. A.; Garcia-Conesa, M. T.; Gomez-Sanchez, M. B.; Garcia-Talavera, N. V.; Gil-Izquierdo, A.; Sanchez-Alvarez, C.; Fontana-Compiano, L. O.; Morga-Egea, J. P.; Pastor-Quirante, F. A.; Martinez-Diaz, F.; Tomas-Barberan, F. A.; Espin, J. C. Occurrence of urolithins, gut microbiota ellagic acid metabolites and proliferation markers expression response in the human prostate gland upon consumption of walnuts and pomegranate juice. *Mol. Nutr. Food Res.* **2010**, *54* (3), 311−322.
(14) Gonzalez-Sarrias, A.; Espin, J. C.; Tomas-Barberan, F. A.; Garcia-Conesa, M. T. Gene expression, cell cycle arrest and MAPK signalling regulation in Caco-2 cells exposed to ellagic acid and its metabolites, urolithins. *Mol. Nutr. Food Res.* **2009**, *53* (6), 686−698.
(15) Lansky, E. P.; Jiang, W.; Mo, H.; Bravo, L.; Froom, P.; Yu, W.; Harris, N. M.; Neeman, I.; Campbell, M. J. Possible synergistic prostate cancer suppression by anatomically discrete pomegranate fractions. *Invest. New Drugs* **2005**, *23* (1), 11−20.
(16) Jemal, A.; Siegel, R.; Ward, E.; Hao, Y.; Xu, J.; Thun, M. J. Cancer Statistics, 2009. *CA Cancer J. Clin.* **2009**, *59* (4), 225−249.
(17) Sporn, M. B.; Newton, D. L. Chemoprevention of cancer with retinoids. *Fed. Proc.* **1979**, *38* (11), 2528−2534.
(18) Hafeez, B. B.; Siddiqui, I. A.; Asim, M.; Malik, A.; Afaq, F.; Adhami, V. M.; Saleem, M.; Din, M.; Mukhtar, H. A dietary anthocyanidin delphinidin induces apoptosis of human prostate cancer PC3 cells in vitro and in vivo: Involvement of nuclear factor-kappaB signaling. *Cancer Res.* **2008**, *68* (20), 8564−8572.
(19) Malik, A.; Afaq, F.; Sarfaraz, S.; Adhami, V. M.; Syed, D. N.; Mukhtar, H. Pomegranate fruit juice for chemoprevention and chemotherapy of prostate cancer. *Proc. Natl. Acad. Sci. U.S.A.* **2005**, *102* (41), 14813−14818.
(20) Longtin, R. The pomegranate: Nature's power fruit? *J. Natl. Cancer Inst.* **2003**, *95* (5), 346−348.
(21) Kim, N. D.; Mehta, R.; Yu, W.; Neeman, I.; Livney, T.; Amichay, A.; Poirier, D.; Nicholls, P.; Kirby, A.; Jiang, W.; Mansel, R.; Ramachandran, C.; Rabi, T.; Kaplan, B.; Lansky, E. Chemopreventive and adjuvant therapeutic potential of pomegranate (*Punica granatum*) for human breast cancer. *Breast Cancer Res. Treat.* **2002**, *71* (3), 203−217.
(22) Albrecht, M.; Jiang, W.; Kumi-Diaka, J.; Lansky, E. P.; Gommersall, L. M.; Patel, A.; Mansel, R. E.; Neeman, I.; Geldof, A. A.; Campbell, M. J. Pomegranate extracts potently suppress proliferation, xenograft growth, and invasion of human prostate cancer cells. *J. Med. Food* **2004**, *7* (3), 274−283.
(23) Chuu, C. P.; Chen, R. Y.; Kokontis, J. M.; Hiipakka, R. A.; Liao, S. Suppression of androgen receptor signaling and prostate specific antigen expression by (−)-epigallocatechin-3-gallate in different progression stages of LNCaP prostate cancer cells. *Cancer Lett.* **2009**, *275* (1), 86−92.
(24) Torres, J. M.; Ruiz, E.; Ortega, E. Development of a quantitative RT-PCR method to study 5alpha-reductase mRNA isozymes in rat prostate in different androgen status. *Prostate* **2003**, *56* (1), 74−79.
(25) Liao, S.; Hiipakka, R. A. Selective inhibition of steroid 5 alpha-reductase isozymes by tea epicatechin-3-gallate and epigallocatechin-3-gallate. *Biochem. Biophys. Res. Commun.* **1995**, *214* (3), 833−838.
(26) Liang, T.; Liao, S. Inhibition of steroid 5 alpha-reductase by specific aliphatic unsaturated fatty acids. *Biochem. J.* **1992**, *285* (Part 2), 557−562.
(27) Hong, M. Y.; Seeram, N. P.; Heber, D. Pomegranate polyphenols down-regulate expression of androgen-synthesizing genes in human prostate cancer cells overexpressing the androgen receptor. *J. Nutr. Biochem.* **2008**, *19* (12), 848−855.
(28) Matias, P. M.; Donner, P.; Coelho, R.; Thomaz, M.; Peixoto, C.; Macedo, S.; Otto, N.; Joschko, S.; Scholz, P.; Wegg, A.; Basler, S.; Schafer, M.; Egner, U.; Carrondo, M. A. Structural evidence for ligand specificity in the binding domain of the human androgen receptor. Implications for pathogenic gene mutations. *J. Biol. Chem.* **2000**, *275* (34), 26164−26171.
(29) Pereira de Jesus-Tran, K.; Cote, P. L.; Cantin, L.; Blanchet, J.; Labrie, F.; Breton, R. Comparison of crystal structures of human androgen receptor ligand-binding domain complexed with various agonists reveals molecular determinants responsible for binding affinity. *Protein Sci.* **2006**, *15* (5), 987−999.
(30) Guo, Q.; Zhao, B.; Shen, S.; Hou, J.; Hu, J.; Xin, W. ESR study on the structure-antioxidant activity relationship of tea catechins and their epimers. *Biochim. Biophys. Acta* **1999**, *1427* (1), 13−23.
(31) Grossmann, M. E.; Mizuno, N. K.; Schuster, T.; Cleary, M. P. Punicic acid is an omega-5 fatty acid capable of inhibiting breast cancer proliferation. *Int. J. Oncol.* **2010**, *36* (2), 421−426.
(32) Nicholson, D. W. Caspase structure, proteolytic substrates, and function during apoptotic cell death. *Cell Death Differ.* **1999**, *6* (11), 1028−1042.
(33) Zimmermann, K. C.; Bonzon, C.; Green, D. R. The machinery of programmed cell death. *Pharmacol. Ther.* **2001**, *92* (1), 57−70.
(34) Reed, J. C. Regulation of apoptosis by bcl-2 family proteins and its role in cancer and chemoresistance. *Curr. Opin. Oncol.* **1995**, *7* (6), 541−546.
(35) 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* (4), 3123−3133.
(36) Uzgare, A. R.; Isaacs, J. T. Enhanced redundancy in Akt and mitogen-activated protein kinase-induced survival of malignant versus normal prostate epithelial cells. *Cancer Res.* **2004**, *64* (17), 6190−6199.
(37) Cross, D. A.; Alessi, D. R.; Cohen, P.; Andjelkovich, M.; Hemmings, B. A. Inhibition of glycogen synthase kinase-3 by insulin mediated by protein kinase B. *Nature* **1995**, *378* (6559), 785−789.
(38) Welsh, G. I.; Wilson, C.; Proud, C. G. GSK3: A SHAGGY frog story. *Trends Cell Biol.* **1996**, *6* (7), 274−279.
(39) Roomi, M. W.; Ivanov, V.; Kalinovsky, T.; Niedzwiecki, A.; Rath, M. Anti-tumor effect of ascorbic acid, lysine, proline, arginine, and epigallocatechin gallate on prostate cancer cell lines PC-3, LNCaP, and DU145. *Res. Commun. Mol. Pathol. Pharmacol.* **2004**, *115*−*116*, 251−264.
(40) Siddiqui, I. A.; Malik, A.; Adhami, V. M.; Asim, M.; Hafeez, B. B.; Sarfaraz, S.; Mukhtar, H. Green tea polyphenol EGCG sensitizes human prostate carcinoma LNCaP cells to TRAIL-mediated apoptosis and synergistically inhibits biomarkers associated with angiogenesis and metastasis. *Oncogene* **2008**, *27* (14), 2055−2063.
(41) Siddiqui, I. A.; Adhami, V. M.; Afaq, F.; Ahmad, N.; Mukhtar, H. Modulation of phosphatidylinositol-3-kinase/protein kinase B- and mitogen-activated protein kinase-pathways by tea polyphenols in human prostate cancer cells. *J. Cell. Biochem.* **2004**, *91* (2), 232−242.
(42) Brusselmans, K.; De Schrijver, E.; Heyns, W.; Verhoeven, G.; Swinnen, J. V. Epigallocatechin-3-gallate is a potent natural inhibitor of fatty acid synthase in intact cells and selectively induces apoptosis in prostate cancer cells. *Int. J. Cancer* **2003**, *106* (6), 856−862.
(43) Hastak, K.; Gupta, S.; Ahmad, N.; Agarwal, M. K.; Agarwal, M. L.; Mukhtar, H. Role of p53 and NF-kappaB in epigallocatechin-3-gallate-induced apoptosis of LNCaP cells. *Oncogene* **2003**, *22* (31), 4851−4859.
(44) Gupta, S.; Ahmad, N.; Nieminen, A. L.; Mukhtar, H. Growth inhibition, cell-cycle dysregulation, and induction of apoptosis by

green tea constituent (−)-epigallocatechin-3-gallate in androgen-sensitive and androgen-insensitive human prostate carcinoma cells. *Toxicol. Appl. Pharmacol.* **2000**, *164* (1), 82−90.

(45) Paschka, A. G.; Butler, R.; Young, C. Y. Induction of apoptosis in prostate cancer cell lines by the green tea component, (−)-epigallocatechin-3-gallate. *Cancer Lett.* **1998**, *130* (1−2), 1−7.

(46) Zhang, Y.; Seeram, N. P.; Lee, R.; Feng, L.; Heber, D. Isolation and identification of strawberry phenolics with antioxidant and human cancer cell antiproliferative properties. *J. Agric. Food Chem.* **2008**, *56* (3), 670−675.

(47) Gil, M. I.; Tomas-Barberan, F. A.; Hess-Pierce, B.; Holcroft, D. M.; Kader, A. A. Antioxidant activity of pomegranate juice and its relationship with phenolic composition and processing. *J. Agric. Food Chem.* **2000**, *48* (10), 4581−4589.

(48) Cerda, B.; Llorach, R.; Ceron, J. J.; Espin, J. C.; Tomas-Barberan, F. A. Evaluation of the bioavailability and metabolism in the rat of punicalagin, an antioxidant polyphenol from pomegranate juice. *Eur. J. Nutr.* **2003**, *42* (1), 18−28.

(49) de Pascual-Teresa, S.; Santos-Buelga, C.; Rivas-Gonzalo, J. C. Quantitative analysis of flavan-3-ols in Spanish foodstuffs and beverages. *J. Agric. Food Chem.* **2000**, *48* (11), 5331−5337.

(50) El-Shaarawy, M. I.; Nahapetian, A. Studies of pomegranate seed oil. *Fette Seifen Austrichmittel* **1983**, *85*, 123−126.

(51) Poyrazoğlu, E.; Gökmen, V.; Artık, N. Organic Acids and Phenolic Compounds in Pomegranates (*Punica granatum* L.) Grown in Turkey. *J. Food Compos. Anal.* **2002**, *15*, 567−575.

Received for review August 25, 2010. Revised manuscript received October 23, 2010. Accepted October 25, 2010. This work was supported financially by a Natural Sciences and Engineering Council (NSERC) of Canada grant (No. 313313) to J.T.S.