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Pomegranate Extracts Potently Suppress Proliferation, Xenograft Growth, and Invasion of Human Prostate Cancer Cells

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ABSTRACT We completed a multicenter study of the effects of pomegranate cold-pressed (Oil) or supercritical CO_2-extracted (S) seed oil, fermented juice polyphenols (W), and pericarp polyphenols (P) on human prostate cancer cell xenograft growth *in vivo,* and/or proliferation, cell cycle distribution, apoptosis, gene expression, and invasion across Matrigel, *in vitro*. Oil, W, and P each acutely inhibited *in vitro* proliferation of LNCaP, PC-3, and DU 145 human cancer cell lines. The dose of P required to inhibit cell proliferation of the prostate cancer cell line LNCaP by 50% (ED_{50}) was 70 μg/mL, whereas normal prostate epithelial cells (hPrEC) were significantly less affected (ED_{50} = 250 μg/mL). These effects were mediated by changes in both cell cycle distribution and induction of apoptosis. For example, the androgen-independent cell line DU 145 showed a significant increase from 11% to 22% in G_2/M cells ($P < .05$) by treatment with Oil (35 μg/mL) with a modest induction of apoptosis. In other cell lines/treatments, the apoptotic response predominated, for example, in PC-3 cells treated with P, at least partially through a caspase 3-mediated pathway. These cellular effects coincided with rapid changes in mRNA levels of gene targets. Thus, 4-hour treatment of DU 145 cells with Oil (35 μg/mL) resulted in significant 2.3 ± 0.001-fold (mean ± SEM) up-regulation of the cyclin-dependent kinase inhibitor $p21^{(waf1/cip1)}$ ($P < .01$) and 0.6 ± 0.14-fold down-regulation of c-myc ($P < .05$). In parallel, all agents potently suppressed PC-3 invasion through Matrigel, and furthermore P and S demonstrated potent inhibition of PC-3 xenograft growth in athymic mice. Overall, this study demonstrates significant antitumor activity of pomegranate-derived materials against human prostate cancer.

KEY WORDS: • ***apoptosis*** • ***chemoprevention*** • ***flavonoid*** • ***p2 1$^{(waf/cip1)}$*** • ***phytoestrogen*** • ***prostate cancer*** • ***punicic acid***

INTRODUCTION

PROSTATE CANCER is one of the most common cancers in European and American males and has a high mortality rate. Treatment options are limited, and there is an urgent need for sustained, focused, and better-tolerated therapies, especially to combat high-grade hormonally insensitive disease.

The underlying causes of sporadic prostate cancer remain poorly understood, but appear multifactorial, and include environmental and genetic factors. Epidemiological studies have associated increased cancer incidence with reduced consumption of fruit, vegetables, and oily fish and relatively high intake of red meat.[1,2] To date, a number of dietary extracts have demonstrated potent growth inhibitory activity against prostate cancer cells.[3–8] Thus for prostate cancer and other indolent tumors, chemopreventive dietary intervention may prove significant for reducing and stabilizing tumor burden.

Pomegranate (*Punica granatum*) is extensively referenced in medical folklore.[9] Its various extracts contain a rich complement of steroidal and related polyphenolic compounds, some of which (*e.g.,* luteolin, kaempferol, quercetin) are estrogenic.[10] Pomegranate pericarp is also very rich in tannins (gallic acid, ellagic acid), which, though not estrogenic, are strongly antioxidant.[11] Both classes of compounds inhibit carcinogenesis[12] and possess additional anticancer properties.[5,13–15] The tannins that appear in commercially processed pomegranate juice from pressing the whole fruit and the peels also augment the juice's antioxidant power.[16]

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Pomegranate seed oil is rich in steroids and sterols, including estrone, 17-α-estradiol, campestrol, estriol, testosterone, stigmasterol, and sitosterol.[13,17] Some of these have cancer chemopreventive properties, including promotion of apoptosis.[18] Remarkably, the oil consists of 80% punicic acid, an extremely rare C18 octadecatrienoic fatty acid and known inhibitor of prostaglandin biosynthesis.[19] Further, polyphenols in the oil inhibit cyclooxygenase and lipoxygenase,[20] an important element of pomegranate seed oil's cancer chemopreventive and therapeutic potential.[21] Pomegranate seed oil inhibits skin cancer carcinogenesis and promotion in mice.[22]

We studied the effects of the pomegranate fractions on three well-characterized human prostatic tumor cell lines *in vitro,* LNCaP, PC-3, and DU 145. Each of these has certain advantages as a model (Table 1).[23] LNCaP is the only cell line with functional androgen receptors, the only one that is androgen sensitive, and that secretes prostate-specific antigen. PC-3 is the only line with real metastatic potential and was selected to study invasion *in vitro* and xenograft growth *in vivo*.

MATERIALS AND METHODS

Preparation of pomegranate fruit (Punica granatum) *extracts*

Pomegranate juice was expeller pressed from Kibbutz Sde Eliahu 1999 "Wonderful" pomegranates, fermented, and then extracted with ethyl acetate to yield the fermented juice polyphenols (W) according to the method previously described.[20] Pericarp polyphenols (P) were similarly derived from the aqueous pericarp extract,[13] and both the P and W fraction were prepared as stock concentrations of 100 mg/mL in ethanol.

After being separated manually from the pericarps following juice extraction, the hard white pomegranate seeds were washed well in running water and dried in a solar convection drier for 48 hours. The seeds were additionally processed to yield Oil by extrusion,[20] or seed oil by supercritical CO_2 extraction (S).[13] For *in vitro* use, the Oil was dissolved dropwise in stirred, heated ethanol to give a stock concentration of 8.25 mg/mL.

Cell culture

The prostate cancer cells, LNCaP [FGC], PC-3, and DU 145 were obtained from American Type Culture Collection (ATCC, Rockville, MD), and cultured according to the provider's instructions. The human prostatic stromal cells (hPCPs) were isolated from patients with benign prostate hyperplasia as described previously[24] and cultivated in the same medium as LNCaP. Normal human prostatic stromal (hPrSC) and epithelial (hPrEC) cells were purchased from Clonetics (Wokingham, UK) and cultured according to the manufacturer's protocol. Cultures were maintained in a humidified incubator at 37°C in 5% CO_2.

Proliferation assays

Two different proliferation assays were used in this study: one the ATP assay, and the second employing amidoblack, the latter allowing rapid screening of agent potency.

Bioluminescent ATP proliferation assay

Dose responses of all three agents were assessed in LNCaP, PC-3, and DU 145 cells by measuring cellular ATP. The cell proliferation assay (ViaLight HS, LumiTech, Nottingham, UK) was used according to the manufacturer's instructions under previously optimized conditions.[25] Briefly, cells were plated into 96-well, white-walled, tissue culture treated plates (2×10^3 cells/well; Fisher Scientific Ltd., Loughborough, UK). The cells were exposed to treatments in a final volume of 100 μL per well with media, and incubated for 96 hours, having been re-dosed after 48 hours. Following the incubation period, 100 μL of nucleotide releasing reagent was added, and the cells were left for 30 minutes at room temperature to extract the ATP. Liberated ATP was quantified through addition of 20 μL of ATP monitoring reagent (containing luciferin and luciferase) using a micro plate luminometer (Berthold Detection Systems, Fisher Scientific Ltd.).

Amidoblack proliferation assay

Amidoblack assays were performed, as described previously,[26] with hPCPs, hPrEC, hPrSC, and LNCaP cells, grown in culture for 1–7 days. For experiments, hPCPs cells were used between passages 6 and 9. All cell types were seeded at 1×10^3 cells per well in clear 96-well plates (Falcon, Heidelberg, Germany). After incubation with the various treatments, cells were fixed (10% formaldehyde, 9% acetic acid, and 0.1 *M* sodium acetate) for 15 minutes followed by staining (0.1% naphthol blue black, 9% acetic acid, and 0.1 *M* sodium acetate) for 30 minutes, all at room temperature. After washing with water, the wells were either

Table 1. Selected Features of Prostate Cancer Cell Lines[22]

Feature	*LNCaP*	*PC-3*	*DU 145*
1. Androgen receptors and androgen sensitivity	+	−	−
2. Secretion of prostate-specific antigen	+	−	−
3. Invasive, high metastatic potential	−	+	−

Table 2. Summary of Antiproliferative Effects (ED_{50}) Mediated by Pomegranate Extracts on Normal, Benign, and Malignant Prostate Cancer Cells

	ED_{50} ($\mu g/mL$)		
Cell line	*P*	*W*	*Oil*
DU 145[a]	30	45	30
PC-3[a]	70	100	50
LNCaP[a,b]	70	100	20
hPCPs[b]	300	150	ND
PrEC[b]	250	50	ND
PrSC[b]	250	50	ND

Cell proliferation in the presence or absence of P, W, and Oil pomegranate extracts was examined by ATP proliferation assays[a] after 96 hours and amidoblack proliferation assays[b] after 168 hours according to Materials and Methods. Each experiment utilized at least triplicate wells and was repeated three times. The data were used to construct dose–response curves, and the doses required to inhibit cell proliferation by 50% [ED_{50} ($\mu g/mL$)] were interpolated from the curves. ND = not determined.

photographed, or dye was eluted with 50 m*M* NaOH, and absorbance was determined at 620 nm.

Cell cycle analysis

The effect of P, W, or Oil on the cell cycle was measured by staining DNA with propidium iodide (PI). Briefly, T25 flasks were seeded with 2.5×10^5 cells and left to adhere overnight to ensure that exponentially proliferating cells were exposed to therapeutic doses of agent (ED_{50} value; Table 2). After 24 and 72 hours, with repeat dosing at 48 hours, total cells, inclusive of those in the medium and adherent to the plastic, were harvested and counted, and 1×10^6 cells were stained with PI buffer [10 mg/mL PI, 1% (wt/vol) trisodium citrate, 0.1% (vol/vol) Triton X-100, and 100 m*M* sodium chloride] for 30 minutes. Cell cycle distribution was determined with a Becton-Dickinson (BD Biosciences–Discovery Labware, BD UK Ltd., Oxford, UK) Flow Cytometer and CellFIT Cell-Cycle Analysis software.

JC-1 staining of apoptotic cells

Induction of apoptosis by P, W, or Oil was studied in a similar manner to the cell cycle analysis. Briefly, 2×10^5 cells were plated into 6-well plates containing coverslips and left to adhere overnight prior to treatment with agent. Following treatment, slides were washed in phosphate-buffered saline (PBS) and incubated in 2 mL of media with JC-1 (2 μg/mL; Molecular Probes, Eugene, OR) for 30 minutes, washed in PBS again, and then fixed in 1 mL of paraformaldehyde (4%). The slips were again washed in PBS, removed, and placed, cell side down, on 10 μL of mountant on glass slides and photographed using fluorescent microscopy. The cells were also quantitatively analyzed with a fluorescence-activated cell sorter with an excitation wavelength at 488 nm and emission wavelength at 530 nm for green fluorescence and 585 nm emission for red fluorescence.

Measurement of caspase 3 activity

PC-3 and DU 145 cells were plated as above and treated with P (50 μg/mL). Cells both adherent and detached were harvested and pelleted, washed in PBS, transferred to an Eppendorf tube, and re-pelleted at 7,500 *g* for 5 minutes at 4°C. The pellet was removed and resuspended in 2 volumes (30 mL) of extraction buffer [20 m*M* HEPES-KOH (pH 7.5), 10 m*M* KCl, 1.5 m*M* $MgCl_2$, 1 m*M* EDTA, 1 m*M* EGTA, and 1 m*M* dithiothreitol] with protease inhibitors. The cells were allowed to swell on ice under hypotonic conditions for 15 minutes and disrupted with a sonicator for 10 seconds. Lysis was confirmed by examination of a small aliquot of the suspension under a light microscope. The disrupted samples were then centrifuged at 15,000 *g* for 15 minutes at 4°C. The supernatant was removed while avoiding disruption of the pellet. To 20 mL of the supernatant, 180 mL of reaction buffer [50 m*M* HEPES (pH 7.4), 75 m*M* NaCl, 0.1% CHAPS, and 2 m*M* dithiothreitol] and 22 mL of caspase substrate (1.5 mg/mL) (Alexis Biochemicals, Bingham, UK) were mixed in a glass tube and incubated for 30 minutes at 37°C. The reaction was stopped by the addition of 500 mL of ice-cold distilled H_2O, and the absorbance was determined at 400 nm against a blank containing buffer and no extract. Caspase activity was subsequently normalized to lysate protein concentration, and the fold increases were compared with control (non-treated) lysates.

Extraction of RNA and reverse transcription (RT)

Cells (1.5×10^6 cells) were seeded into T75 flasks, allowed to grow for 36 hours, and treated for 4 hours with the same concentration of agent as used in the cell cycle analysis. Total RNA was extracted using the GenElute RNA system (Sigma, St. Louis, MO). cDNA was prepared from 1 μg of total RNA by RT with Mu-MLV (Promega, Southampton, UK) at 42°C for 60 minutes in the presence of 100 m*M* Tris-HCl (pH 9.0), 500 m*M* KCl, 2 m*M* $MgCl_2$, 100 p*M* random hexamers (Pharmacia, Pisacataway, NJ), 2 m*M* deoxynucleotide triphosphates, and 20 U of RNAsin (Promega) in a 20-μL reaction volume.

Real-time quantitative polymerase chain reaction (PCR)

Expression of specific cDNAs was quantified using the ABI PRISM 7700 Sequence Detection System (Applied Biosystems, Foster City, CA). Each sample was amplified in triplicate wells in 25-μL volumes containing 1× TaqMan Universal PCR Master Mix [3 m*M* $Mn(OAc)_2$, 200 μM deoxynucleotide triphosphates, 1.25 units of AmpliTaq Gold polymerase, and 1.25 units of AmpErase UNG], 3.125 pmol of FAM-labeled TaqMan probe, and 22.5 pmol of primers. All reactions were multiplexed with pre-optimized control

primers and VIC (a fluorescent dye)-labeled probe for 18S ribosomal RNA (PE Biosystems, Warrington, UK). Target gene primers and probes sequences were synthesized by Oswell Scientific (Southampton, UK) and are given in Table 3. Reactions were cycled as follows: 50°C for 2 minutes, 95°C for 10 minutes; then 44 cycles of 95°C for 15 seconds and 60°C for 1 minute. Data were expressed as *Ct* values (the cycle number at which logarithmic PCR plots cross a calculated threshold line) and used to determine ΔCt values ($\Delta Ct = Ct$ of the target sequence minus Ct of the housekeeping sequence). The data were transformed through the equation $2^{-}\Delta\Delta^{Ct}$ to give fold changes in gene expression. To exclude potential bias due to averaging of data all statistics were performed with ΔCt values. Measurements were carried out a minimum of three times each in triplicate wells.

Cell invasion through Matrigel

The *in vitro* invasion assay was performed as described previously.[27] Briefly, transwell chambers (Costar, Cambridge, MA) equipped with 6.5-mm-diameter polycarbonate membrane (pore size 8 μm) were precoated with a solubilized tissue basement membrane (Matrigel, Collaborative Research Products, Bedford, MA; 50 μg per membrane). After gel rehydration, 5×10^4 prostate cancer cells were added to each chamber. Hepatocyte growth factor/scatter factor (40 ng/mL) was used as an invasion inducer. The upper chamber contained P, W, or Oil at 3 μg/mL. After 72 hours of culture, the invasive cells stuck to the lower surface were fixed and stained with crystal violet. The number of cells was quantified using an inverted microscope and expressed as a percentage of the positive control.

Antitumor activity of pomegranate extracts in mice

Effects of supercritically extracted pomegranate seed oil (S) and P were tested on the growth of PC-3 xenografts in athymic BALB/c male homozygous nude mice with an age ranged between 42 and 56 days old. Cryopreserved solid tumors (4 mm^3) were implanted subcutaneously at the right flank region to give initial PC-3 tumors. Treatment or control solution (100 μL) was injected subcutaneously 5–10 minutes before injection of cells. Control mice were treated with PBS containing the same dose of diluent as treatments. Treatments were in the same carrier with S or P (2 μg/g of body weight). Results from treatment groups were compared with those of control.

Statistical analysis

All analyses were compared using Student's *t* test.

RESULTS

Figure 1 depicts the responses of LNCaP, PC-3, and DU 145 cells to P (Fig. 1A), W (Fig. 1B), and Oil (Fig. 1C) using the ATP bioluminescent proliferation assay. All agents were potent inhibitors of proliferation, with cell line DU 145 being most sensitive to P and W (Fig. 1A and B), and LNCaP being relatively most sensitive to Oil (Fig. 1C). The dose of each agent required to inhibit cell proliferation by 50% (ED_{50} value) was interpolated from dose–response curves (Table 2). For example, for P the ED_{50} values were 70 μg/mL for LNCaP, 70 μg/mL for PC-3, and 30 μg/mL for DU 145. Sensitivity to W was also most acute in DU 145 cells. Treatment of cells with a fixed dose (50 μg/mL) of either P or W resulted in significantly greater inhibition of DU 145 cell growth than LNCaP or PC-3 cells. For example, treatment with this dose of W resulted in $40 \pm 4\%$ (mean $\pm$ SEM growth as a percentage of control) in DU 145, but $88 \pm 8\%$ and $83 \pm 6\%$ in PC-3 and LNCaP, respectively ($P < .001$) (data not shown).

The relative sensitivity of LNCaP cells, their normal epithelial equivalent (hPrEC), hyperplastic stromal cells (hPCPs), and their normal equivalent (hPrSC) to extracts W

Table 3. Sequences of Real-Time RT-PCR Primers and Probes

Primer	*Sequence*
c-myc	
Forward primer	TCAAGAGGTCCACGTCTCC
Reverse primer	TCTTGGCAGCAGGATAGTCCTT
Probe	CAGCACAACTACGCAGCGCCTCC
GADD45	
Forward primer	AAGACCGAAAGGATGGATAAGGT
Reverse primer	GTGATCGTGCGCTGACTCA
Probe	TGCTGAGCACTTCCTCCAGGGCAT
MAPK-APK2	
Forward primer	GCCTGCTGATTGTCATGGAA
Reverse primer	TGGTCTCCTCGATCCTGGAT
Probe	TTTGACGGTGGAGAACTCTTTAGCCGT
P21	
Forward primer	GCAGACCAGCATGACAGATTTC
Reverse primer	GGATTAGGGCTTCCTCTTGGA
Probe	CCACTCCAAACGCCGGCTGATCTTT

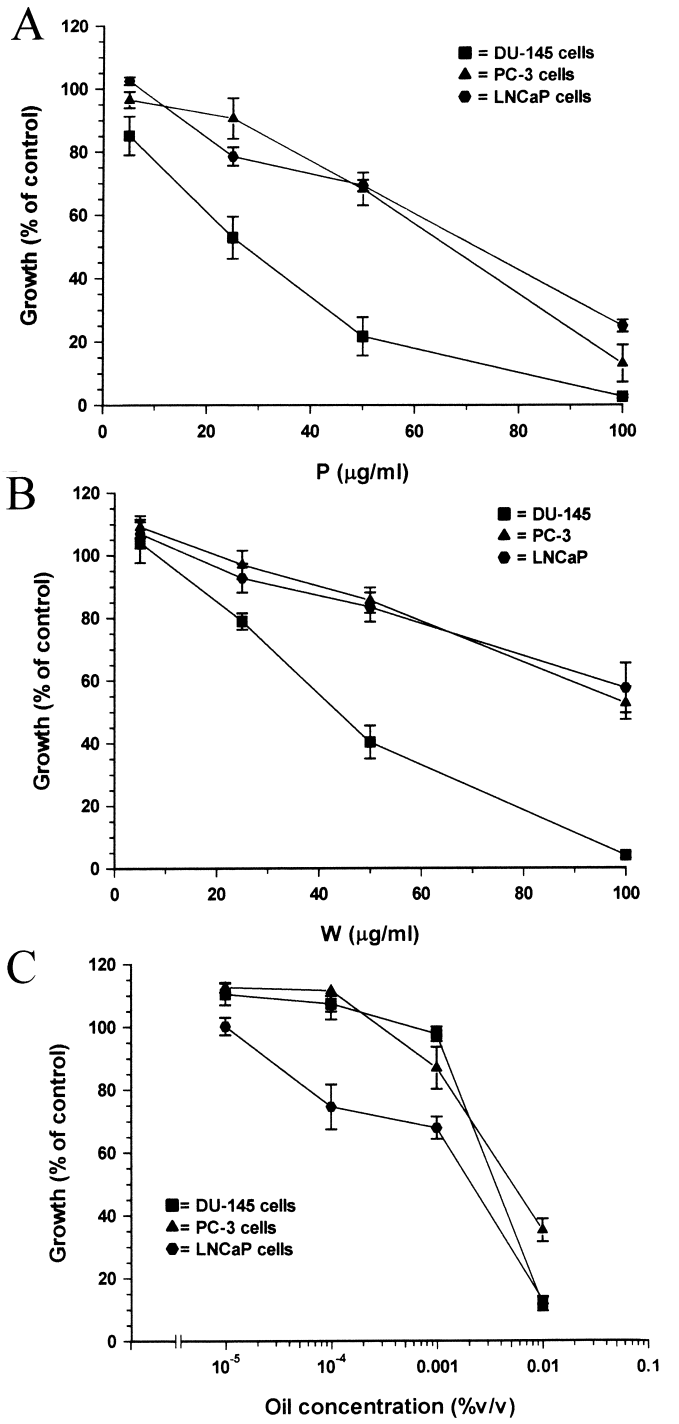

FIG. 1. Growth response curves of DU 145, PC-3, and LNCaP human prostate cancer cells to treatment with the pomegranate fractions pericarp polyphenols (P) (**A**), fermented juice polyphenols (W) (**B**), and cold-pressed seed oil (Oil) (**C**). Each point represents the mean (± SEM) of triplicate wells. Experiments were undertaken three times.

(data not shown) and P (Fig. 2) was quantified using the amidoblack assay. The suitability of the amidoblack assay for pictorial demonstration is evident in Figure 3, which employs photomicrography to compare proliferation of LNCaP cells treated with W and P to the proliferation of the homologous normal epithelial cells, hPrEC, treated with the same extracts. The cancerous LNCaP cells were considerably more sensitive to these extracts than the non-cancerous cells.

Changes in cell cycle distribution in DU 145 cells treated with the extracts are depicted in Figure 4. No significant changes in cell cycle were seen after a 24-hour treatment, but after a 72-hour treatment, with a re-treatment after 48 hours, significant changes were detectable. These changes included a decrease in the percentage of cells in G_1, relative to control, following treatment with P and W (30 and 45 μg/mL, respectively). All three agents, however, resulted in significant increases in G_2/M ($P < .05$). By contrast, PC-3 cells did not show any significant changes in cell cycle profile distribution after either a 24- or 72-hour treatment (not shown).

The number of apoptotic cells was evaluated by staining with JC-1 dye as a marker of mitochondrial membrane polarity. In the mitochondria of healthy cells the JC-1 dye aggregates and fluoresces red against a backdrop of green monomeric cytoplasmic staining. In apoptotic cells the dye cannot accumulate in the mitochondria and therefore reveals a reduction in red staining. Figure 5A shows the number of PC-3 cells exposed to the aqueous compartment extracts P and W that displayed a loss of red staining and an increase in mitochondrial membrane permeability. Figure 5B shows the changes in PC-3 cells by photomicrography. Similar results were seen in DU 145 cells treated with the pomegranate extracts (data not shown). Treatment of both cell lines with P (50 μg/mL) for 72 hours resulted in a mean 9.4 ± 1.3- and 5.3 ± 0.9-fold increase in caspase 3 activity in PC-3 and DU 145 cells, respectively ($P < .001$).

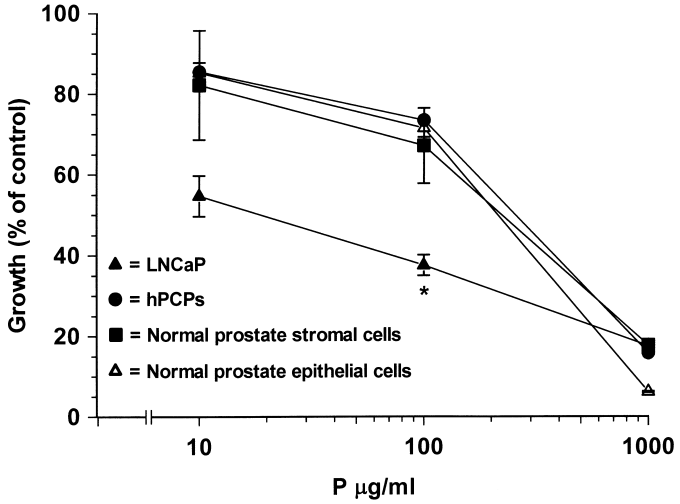

FIG. 2. Growth response of four human prostate cell lines—LNCaP, androgen-sensitive prostate cancer cells; hPCPs, hyperplastic stromal cells; hPrSC, normal prostatic stromal cells; and hPrEC, normal prostatic epithelial cells—to treatment with pomegranate pericarp polyphenols (P). Each point represents the mean ± SEM of triplicate wells. Experiments were performed three times.

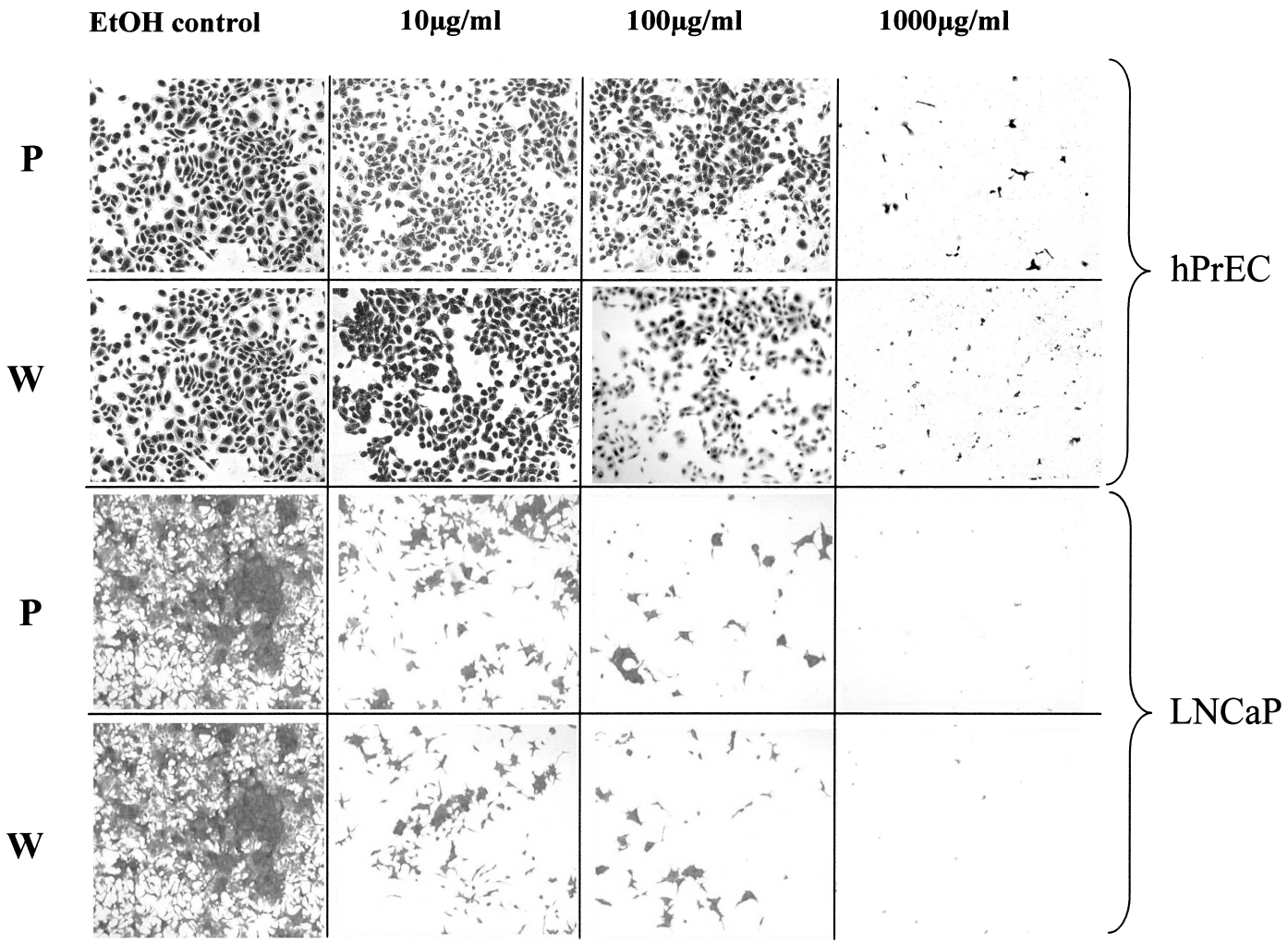

FIG. 3. Photomicrographs of LNCaP prostate cancer cells and their normal homologues, hPrEC (normal prostate epithelial cells), after treatment with pomegranate pericarp polyphenols (P) and pomegranate fermented juice polyphenols (W). LNCaP cells and their normal counterparts were photographed after incubation with P and W and staining with naphthol blue black solution. Compared with normal epithelial prostatic cells (PrEC), the LNCaP prostate tumor cells are more sensitive to the growth-inhibiting effect of the extracts P and W. EtOH, ethanol. Magnification ×200.

We reasoned that the changes in cell cycle and apoptosis profiles resulted from changes in gene expression that occurred at earlier time points. Therefore, the regulation of several gene targets in response to a 4-hour treatment with the ED_{50} concentrations of P, W, and Oil, respectively, was examined in PC-3 and DU 145 cells. The genes were chosen for their role in initiating cell cycle arrest, differentiation, and/or apoptosis. Agent-driven changes in gene expression for certain cell line/treatment combinations were observed, suggesting potent, selective transactivational activities (Table 4). Analysis of the cyclin-dependent kinase inhibitor (CDKI) $p21^{(waf/cip1)}$ showed a significant 2.05 ± 0.05-fold (mean ± SEM) change ($P < .05$) in expression in PC-3 cells after treatment with W (100 μg/mL) and 2.32 ± 0.001-fold change ($P < .01$) in DU 145 cells treated with Oil (35 μg/mL). Similarly mitogen-activated protein kinase (MAPK)-APK2 was up-regulated (1.57 ± 0.002-fold; $P < .01$) in DU 145 cells treated with Oil (35 μg/mL).

The effect on gene regulation was not only positive, but also included negative effects. For example, both PC-3 and DU 145 cells treated with Oil displayed significant down-regulation in expression of c-myc (0.39 ± 0.03- and 0.63 ± 0.14-fold, respectively; $P < .01$)]. Similarly, GADD45α was also down-regulated by all treatments in PC-3 cells, most dramatically, with Oil (40 μg/mL), resulting in a 0.0002 ± 0.0008-fold decrease ($P < .0001$). A similar significant down-regulation (0.18 ± 0.07-fold) was observed in DU 145 cells treated with Oil (35 μg/mL; $P < .001$).

All agents significantly inhibited invasion of PC-3 cells through Matrigel (Fig. 6). For example, treatment with P (3 μg/mL) significantly suppressed the ability to migrate through the Matrigel membrane, by 36 ± 4%, compared

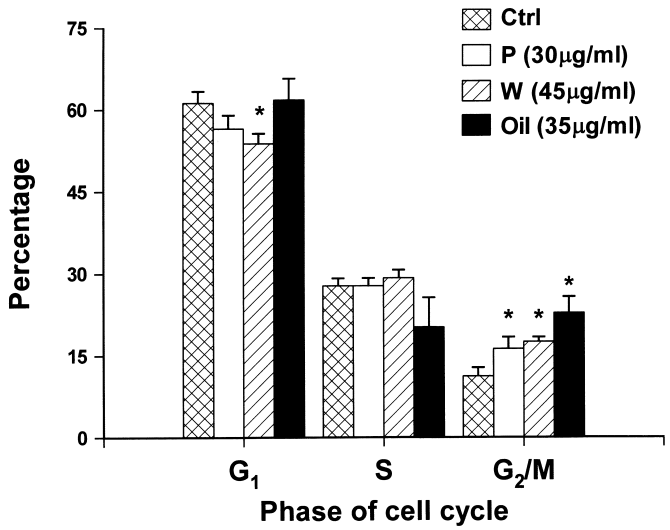

FIG. 4. Effect of pomegranate pericarp polyphenols (P), fermented juice polyphenols (W), and seed oil (Oil) on cell cycle distribution of DU 145 human prostate cancer cells. Midexponentially proliferating cells were exposed to the various extracts P, W, and Oil at 30, 45, and 35 μg/mL, respectively, for 72 hours (re-dosed after 48 hours), harvested, and stained with PI. Fluorescence-activated cell sorter analyses revealed changes in population cell cycle distribution in response to the treatments. Incubating the cells with the P, W or Oil extract leads to a shift towards the G_2/M phase of the cell cycle. Ctrl, control. Each point represents the mean of at least three experiments with triplicate dishes. $*P < .05$.

with hepatocyte growth factor/scatter factor positive controls ($P < .01$).

The potencies of P and S were also assessed *in vivo* using a subcutaneous PC-3 xenograft model in athymic mice. The mice were randomized into three groups (five mice in each group) and treated with either P or S, each at 2 μg/g of body weight, or left untreated according to Materials and Methods. After 35 days the tumor volume was measured in the four groups (Table 5). Both P and S significantly inhibited growth of tumors. There were no detectable differences in survivor fractions between test and control groups.

DISCUSSION

In the study presented, we examined the activities of four extracts of the pomegranate fruit (*P. granatum*), namely, pomegranate pericarp polyphenols (P), pomegranate fermented juice polyphenols (W), cold pressed pomegranate seed oil (Oil), and seed oil extracted by supercritical CO_2 extraction (S). Chemical analysis conducted by an independent laboratory did not show significant variation in the chemical profiles of the pomegranate seed oil obtained from the same pomegranate source by the two different extraction methods (data not shown).

We studied the effects of the extracts on cell proliferation, cell cycle distribution, apoptosis, gene regulation, invasion and *in vivo* tumor growth. All the extracts were potent inhibitors of cancer cell proliferation, and, in the case of P, the normal and benign prostate epithelial cells displayed reduced sensitivity, compared with cancer cells.

For mechanistic studies we focused on the androgen-independent PC-3 and DU 145 cells and found that all three agents induced G_2M arrest, associated with loss of mito-

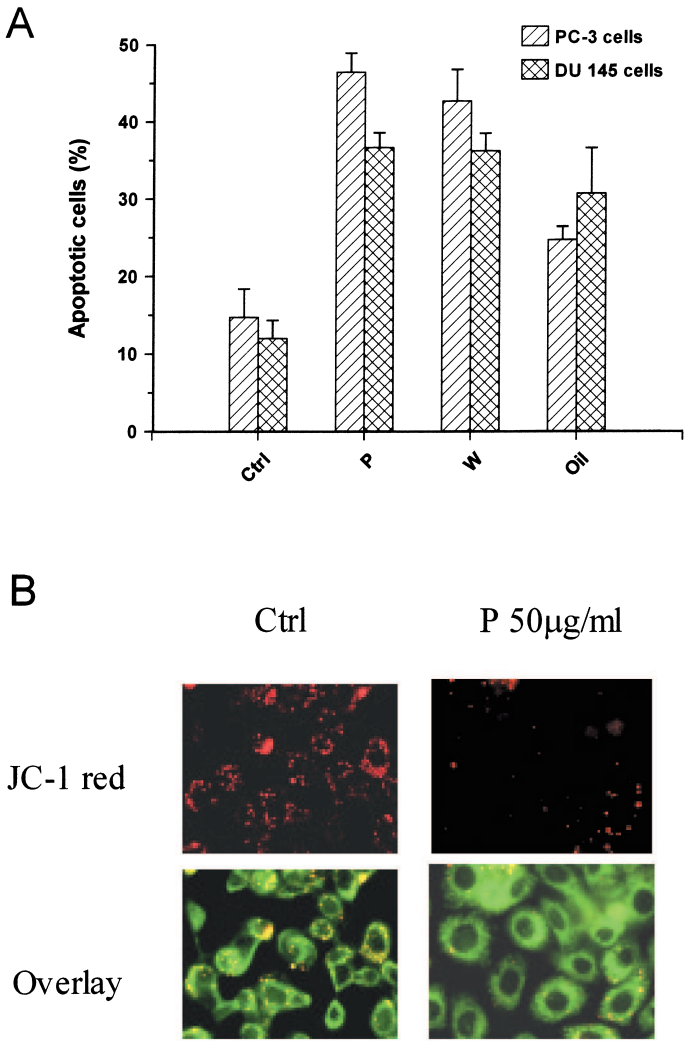

FIG. 5. Induction of apoptosis by pomegranate extracts in PC-3 cells. (**A**) Mid-exponentially proliferating PC-3 cells and DU 145 cells were exposed to the various extracts P, W, and Oil at 70, 100, and 50 μg/mL, respectively, for 72 hours (re-dosed after 48 hours), harvested, and stained with JC-1 dye. Fluorescence-activated cell sorter analyses revealed an increase in the number of apoptotic cells following treatment with various extracts. As can be noted, P and W had the strongest pro-apoptotic effect in the PC-3 cells, with a somewhat milder effect in the DU 145 cells. An overall milder proapoptotic effect was caused by Oil, though, relatively, Oil stimulated apoptosis more strongly in DU 145 cells than in PC-3 cells. (**B**) PC-3 cells were allowed to adhere to glass coverslips and treated with P, W, or Oil. Ctrl, control. Cells were re-dosed after 48 hours and harvested after 96 hours. Mitochondrial membrane integrity was measured by staining with JC-1 dye. Cells undergoing apoptosis lose mitochondrial membrane integrity, and the mitochondria do not fluoresce red; instead, general green staining is observed in the cytoplasm. Representative images are shown at ×400 magnification.

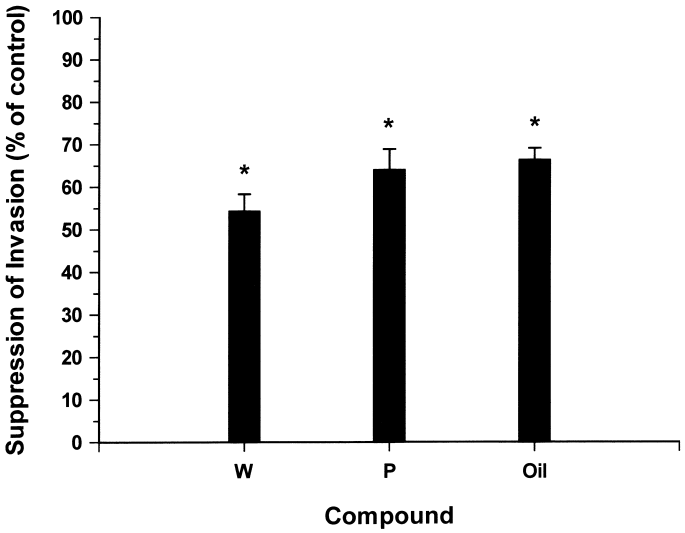

FIG. 6. Inhibition of PC-3 cell invasion through a Matrigel membrane by pomegranate extracts. The effect of P, W, or Oil (3 μg/mL) on hepatocyte growth factor-stimulated PC-3 invasion through a precoated membrane was investigated. After 72 hours in culture, invasive cells were fixed and stained with crystal violet. The number of cells was quantified using an inverted microscope. Invasion is expressed as percentage of the positive control (hepatocyte growth factor). Each point represents the mean of at least three experiments with triplicate dishes. *$P < .05$.

chondrial membrane integrity, which is indicative of apoptosis. Further, at least in the case of P, this apoptosis is mediated at least partially through a caspase 3-mediated pathway. These data, and those of others, have shown that divergent phytochemical agents induce G_2M cell cycle arrest. For example, genistein-treated breast cancer cell lines display a G_2M arrest, associated with suppression of cyclin B expression, in a p53-independent manner.[4,28,29] This is reflected by the current study as both PC-3 and DU 145 cells have abrogated p53 function.[30] Similarly, numerous other phytoestrogens and flavonoids also induce apoptosis; for example, green tea catechin, a flavonol, and baicalin, one of the active components in PC-SPES, both induce apoptosis in DU 145.[6,8,31]

To dissect the mechanisms of action further, we examined the regulation of some target genes known to play a role in cell cycle regulation and apoptosis. These studies revealed that short-term exposure of cells to the various agents regulated mRNA expression of several targets. For example, $p21^{(waf/cip1)}$ mRNA was up-regulated in PC-3 cells treated with W, and DU 145 cells treated with Oil. Similarly, up-regulated $p21^{(waf/cip1)}$ expression has been shown in response to genistein in breast cancer cell lines, leading to G_2M arrest.[28,32] We also examined expression of GADD45α, which interacts with $p21^{(waf/cip1)}$ and other cell cycle regulators to facilitate cell cycle arrest, DNA surveil-

TABLE 4. SUMMARY OF FOLD CHANGES IN GENE EXPRESSION MEDIATED BY POMEGRANATE EXTRACTS ON PROSTATE CANCER CELLS

	p21	*c-myc*	*GADD45α*	*MAPK-APK2*
PC-3				
P	1.32 ± 0.22	1.01 ± 0.09	*0.16 ± 0.02*	1.06 ± 0.20
W	*2.05 ± 0.05*	1.07 ± 0.08	*0.004 ± 0.01*	1.26 ± 0.44
Oil	1.10 ± 0.70	*0.39 ± 0.03*	*2×10^{-4} ± 0.008*	0.94 ± 0.36
DU 145				
P	1.41 ± 0.16	1.22 ± 0.27	0.77 ± 0.47	1.07 ± 0.52
W	1.33 ± 0.35	1.19 ± 0.08	1.32 ± 0.23	0.96 ± 0.28
Oil	*2.32 ± 0.001*	*0.63 ± 0.14*	*0.18 ± 0.07*	*1.57 ± 0.002*

Levels of the target genes were quantified using real-time RT-PCR. PC-3 and DU 145 cells were exposed to the agent at the evaluated ED_{50} value for 4 hours compared with untreated (control) cultures. Total RNA was extracted and reverse transcribed, and for each cell line the fold change between treatment and untreated control was calculated according to Materials and Methods. Each experiment utilized triplicate wells and was repeated three times. Italic numbers indicate statistically significant values ($P < 0.01$).

TABLE 5. SUPPRESSION OF PC-3 XENOGRAFT GROWTH BY PHYTOCHEMICALS

Treatment period	*Control*	*S (2 μg/g of body weight)*	*P (2 μg/g of body weight)*
Day 0	4 mm^3	4 mm^3	4 mm^3
Day 35	194 ± 6 mm^3	*55 ± 2 mm^3*	*55 ± 1 mm^3*

Effects of supercritically extracted pomegranate seed oil (S) and pomegranate pericarp polyphenols (P) were tested on growth of PC-3 xenografts implanted subcutaneously as solid tumors (4 mm^3) into athymic BALB/c male homozygous nude mice. Treatment was undertaken as described in Materials and Methods. The mean results from treatment groups were compared with those for appropriate controls. Italic numbers indicate the differences in the intergroup means were significant ($P < 0.001$). There were no detectable differences in survivor fractions between test and control groups.

lance, and initiation of apoptosis.[33,34] However, unexpectedly we observed a rapid and dramatic down-regulation of GADD45α mRNA expression in PC-3 cells with all treatments and in DU 145 cells with Oil. The down-regulation may result in an inability to promote DNA repair and thereby provide an environment permissive of apoptosis. Similarly, down-regulation of c-myc, which favors differentiation and/or apoptosis, was significant after treatment with Oil in both PC-3 and DU 145 cells. Down-regulation of c-myc in DU 145 was also associated with up-regulation of MAPK-APK2 mRNA, another regulator of differentiation and apoptosis subpaths.[35]

In summary, these complex phytochemical mixtures initiate multiple pathways for suppression of proliferation, and specific gene expression profiles combine to determine specific cell fates. Thus, DU 145 cells treated with Oil alter the balance of p21$^{(waf/cip1)}$, MAPK-APK2, and c-myc, which collectively may promote the observed G_2M arrest and apoptosis, and possibly promote differentiation. The multiple pathways illustrate the broad spectrum and modes of action of the pomegranate extracts that collectively result in an inhibition of proliferation.

Subsequently, we examined the effect of pomegranate extracts on the invasive potential of tumor cells. All three agents very potently suppressed *in vitro* invasion through Matrigel; similar anti-invasive capacity has been reported for other phytochemicals.[36] The collective effects of these fractions to control the proliferation of prostate cancer cells were seen most clearly by the ability to significantly inhibit *in vivo* proliferation of PC-3 cells implanted into athymic mice, according to Materials and Methods. Interestingly, 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 (data not shown).

The mechanisms that link diet in general and cancer remain elusive, and we are currently dissecting the signaling mechanisms that mediate the potent anticancer properties described in the current study. Growth of the prostate gland is governed by a complex milieu of hormonal factors signaling, in part, *via* a range of nuclear receptors. The receptors act as a homeostatic mechanism by sensing a diverse range of dietary, xenobiotic, and environmental factors. For example, phytoestrogens and flavonoids are able to regulate estrogen and peroxisome proliferator-activated receptors.[37,38] The pomegranate fractions may target nuclear receptor-mediated gene regulation and thereby regulate cell proliferation.

Translation of the growth inhibitory and anti-invasive capacity of pomegranate extracts observed in this study to the clinic would be dramatic, bearing in mind the indolent nature of the disease, the paucity of therapeutic options, and the capacity for comparatively well-tolerated chronic administration.

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