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Possible synergistic prostate cancer suppression by anatomically discrete pomegranate fractions

Ephraim P. Lansky[1], Wenguo Jiang[2], Huanbiao Mo[3], Lou Bravo[3], Paul Froom[4], Weiping Yu[5], Neil M. Harris[6], Ishak Neeman[7] and Moray J. Campbell[8]

[1]*Rimonest Ltd., Horev Center, Box 9945, Haifa, Israel;* [2]*University Department of Surgery, University of Wales College of Medicine, Cardiff, UK;* [3]*Department of Nutrition and Food Science, Texas Women's University, Denton, TX, USA;* [4]*Department of Epidemiology and Preventive Medicine, Sackler Medical School, Tel Aviv University, Tel Aviv, Israel;* [5]*School of Biological Sciences, University of Texas at Austin, Austin, TX, USA;* [6]*Department of Urology, Royal Bournemouth Hospital, Bournemouth, UK;* [7]*Department of Food Engineering and Biotechnology, Technion—Israel Institute of Technology, Haifa, Israel;* [8]*Division of Medical Sciences, University of Birmingham Medical School, Birmingham, UK*

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Summary

We investigated whether dissimilar biochemical fractions originating in anatomically discrete sections of the pomegranate (*Punica granatum*) fruit might act synergistically against proliferation, metastatic potential, and phosholipase A2 (PLA2) expression of human prostate cancer cells *in vitro*. Proliferation of DU 145 human prostate cancer cells was measured following treatment with a range of therapeutically active doses of fermented pomegranate juice polyphenols (W) and sub-therapeutic doses of either pomegranate pericarp (peel) polyphenols (P) or pomegranate seed oil (Oil). Invasion across Matrigel by PC-3 human prostate cancer cells was measured following treatment with combinations of W, P and Oil such that the total gross weight of pomegranate extract was held constant. Expression of PLA2, associated with invasive potential, was measured in the PC-3 cells after treatment with the same dosage combinations as per invasion. Supra-additive, complementary and synergistic effects were proven in all models by the Kruskal-Wallis non-parametric H test at $p < 0.001$ for the proliferation tests, $p < 0.01$ for invasion, and $p < 0.05$ for PLA2 expression. Proliferation effects were additionally evaluated with CompuSyn software median effect analysis and showed a concentration index CI < 1, confirming synergy. The results suggest vertical as well as the usual horizontal strategies for discovering pharmacological actives in plants.

Introduction

Synergy, *cooperative force* [1], benefits the defense of plants. Thus, endochitinase or exochitinase alone only marginally protects against scab when genes encoding for either are introduced into apple plants from the fungus *Trichoderma atroviride,* but when inserted together, synergistic enhancement of the protection results [2].

Synergy also benefits both cancers and anticancer forces. So, malignant tumor cells cooperate as cellular triplets to vanquish more complex, and consequently less efficient, "cellular cultures" of their hosts [3], and the same hosts' natural killer and cytotoxic T cells synergistically counter-attack, triggering complementary apoptotic mechanisms in the neoplastic clone [4].

Synergy is also advantageous in exogenous anti-cancer strategies. For example, picogram doses of Tumor Necrosis Factor-*alpha*, a cytokine which increases vascular permeability, synergizes with the chemotherapeutic agent doxorubicin to improve the latter's penetration into tumors [5].

Natural products originating in different plants may act cooperatively against cancer. Thus, quercetin (e.g., from onions), and genistein (e.g., from soya) synergistically inhibit proliferation of ovarian carcinoma cells [6]. Further, mixing flavonoids from orange juice with tocotrienols from palm oil synergistically retards breast cancer growth both *in vitro* and *in vivo* [7].

As medicines, adjuvant compounds within single plants may augment the activity of "active principles" [8, 9].

Thus, the antimicrobial action of the alkaloid berberine is enhanced by 5′methoxyhydrocarpin (5′MHC), a compound found in the same plants as berberine, but itself without antimicrobial activity. Apparently, 5′MHC inactivates "multidrug resistance pumps" that normally prevent such antimicrobial agents from accumulating in the bacterial cytoplasm [10].

The present study breaks new ground by describing synergistic actions of phytochemical mixtures from different antatomical compartments of a single plant. The chemistries of the separate compartments are dissimilar and the compounds from each most likely target different signaling pathways within the same cancer cells. Yet the overall effect is more than addition.

Three different anatomically discrete pomegranate "fractions" were used in this study. These will here be briefly described, and their respective and overlapping chemistries briefly elaborated.

a. *Pomegranate fermented juice polyphenols* (W). This is the fraction "W," for "wine," and refers to the ethyl acetate extract of the concentrated, de-alcoholized, fermented pomegranate juice. In general, fermented fruit juice differs from unfermented fruit juice, in part, because certain key flavonoids are in the fresh, unfermented state bound to sugar moieties, as glycosides, galactosides, rhamnosides, etc., but in the fermented state, the flavonoids exist as free compounds since the flavonoid-sugar complexes have been sundered as the sugar components were consumed during fermentation. Further, the entire matrix holding the active compounds has become more concentrated, an effect separate from the greater activity of free flavonoids as opposed to sugar-flavonoid complexes.

Significant overlap exists between the chemistry of W, from the fermented juice, and that of P, to be taken up below, from the peels. In a base consisting of common fruit acids such as citric, malic, lactic, fumaric, tartaric and even acetic [11], the juice also contains numerous, pharmacologically more active, phenolic compounds, most of which occur in even higher concentrations in the peels [12]. These include flavonoids such as quercetin and rutin, phenolic acids such as quinic acid, gallic acid, chlorogenic acid, caffeic acid and ferulic acid, and also ellagotannins such as ellagic acid, and punicalagin [13]. The only phenolic compounds arguably unique to the juice and not the peels are anthyocyanidins, such as delphinidin, but these are not expected to survive much heating or aging. Further, pro-anthocyanidins, specifically pro-delphinidins, have been discovered in the peels, along with gallocatechins [14], all of which are strongly antioxidant.

Thus, for all intents and purposes, the chemistries of the peels (including inner membranes) and juice consist of different blends of much the same active phenolic compounds, deriving from the flavonoids (including flavonols, flavones, flavanones, anthocyanidins) and tannins, ellagitannins and catechins. All of these compounds are potently antioxidant, but only certain flavonoids are known to be estrogenic/anti-estrogenic (and therefore also frequently androgenic/anti-androgenic). Ellagic acid is an extremely potent antioxidant, but it is not active at estrogen receptors [15]. A number of unknown compounds [12] have also been recognized on HPLC traces, at least one of which has cancer chemoprotective activity in a mouse mammary organ culture (MMOC) greater than of the parent fraction W [16].

b. *Pericarp polyphenols* (P). This is an ethyl acetate extract obtained from the simple aqueous extract of the fresh peels. As stated above, the range of active polyphenolic chemicals but not their relative concentrations is comparable. In addition, other polyphenolics with estrogenic activity including kaempferol and luteolin have also been noted in peel extracts [17], but not as yet described in juice. This large grouping of polyphenolic compounds common both to W and P is summarized in Table 1. Structures of some of these compounds are shown in Figure 1.

c. *Seed oil (Oil)*. Whereas the ethyl acetate extracts of the aqueous peel extract and the fermented juice concentrate represent only about 1% of the total mass of solids derived from fermented juice concentrate, on the one hand, or aqueous peel extract, on the other, pomegranate seed oil was used in these studies in its "whole" state following mechanical extrusions, without further extraction. In fact, pomegranate seed oil also contains polyphenolic compounds, though their sum total represents less than 1% of the whole oil. However, these compounds

Table 1. Selected pharmacological actives in pomegranate

In seeds	In juice/peels	MW	Class	Reference
17 α estradiol		272	steroid	19
estrone		270	steroid	20, 51
estriol		288	steroid	20
testosterone		288	steroid	20
coumestrol		268	coumestan	52
campesterol		401	sterol	19
stigmasterol		417	sterol	19, 20
β-sitosterol		415	sterol	19, 20
γ-tocopherol		417	tocopherol	19
punicic acid		278	trienoic acid	18, 19, 22, 46
	luteolin	286	flavone	17
	kaempferol	286	flavonol	17
	quercetin	302	flavonol	12, 17
	naringin	581	flavanone	19
	rutin	611	flavonol	*
	ellagic acid	302	ellagitannin	53, 54
	punicalagin		ellagitannin	43, 44
	caffeic acid	180	phenolic acid	12
	quinic acid	192	phenolic acid	12

For names of compounds, **bold** >**1%**, *italic* >0.01% regular, <0.001%.
* = unpublished data.

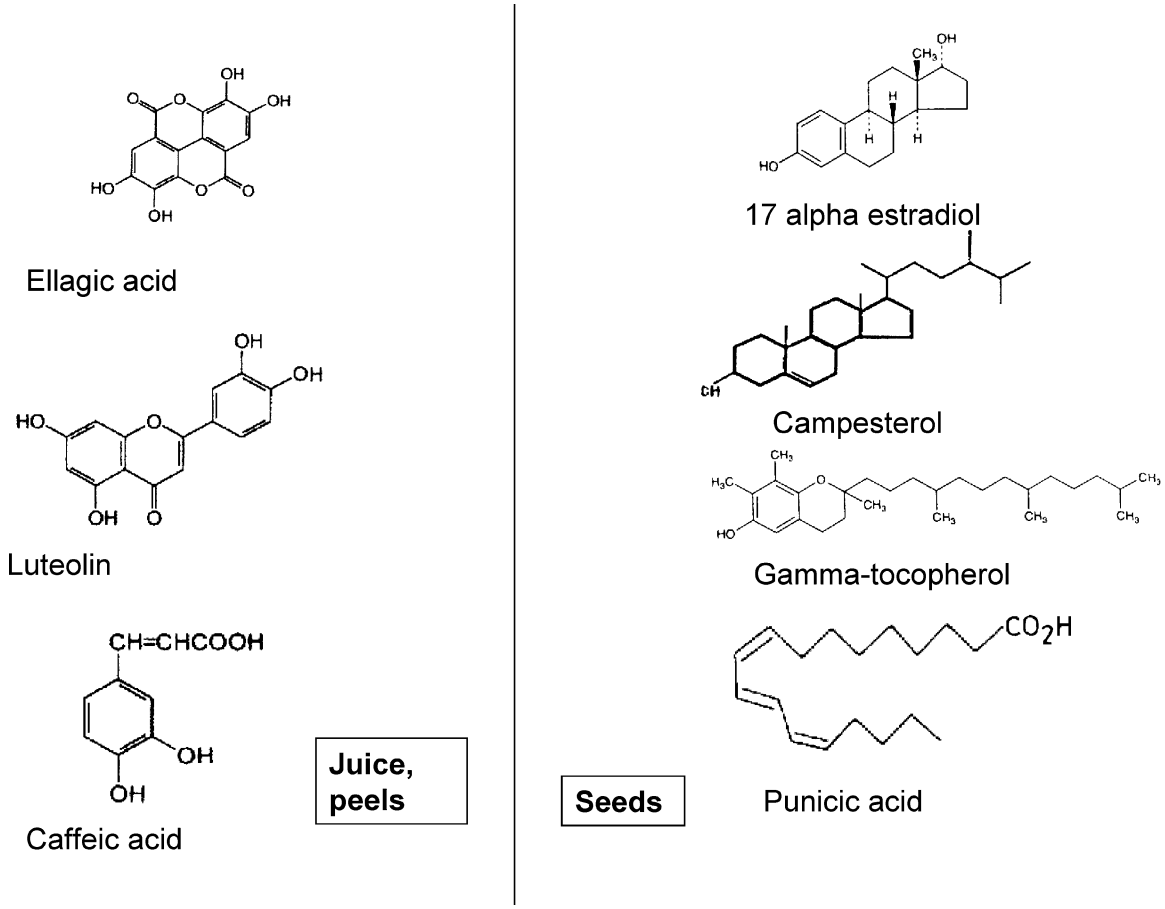

Figure 1. Structures of selected compounds in pomegranate juice/peels and seeds.

are less potent antioxidants than those from the fermented juice [18]. The Oil also contains other compounds of interest with known anti-cancer activities, including sterols (beta-sitosterol, stigmasterol and campesterol), tocopherols (gamma-tocopherol), and steroids (17-alpha estradiol, estrone, estriol) [19, 20]. Most significantly, the oil is comprised of 65–80% conjugated fatty acids, specifically, trienoic fatty acids containing three double bonds, between which alternate individual single bonds. The predominant compound among these is punicic acid (~85% of the total trienoic acids), which occurs along with three isomers (~15% of the trienoic acids). Trienoic fatty acids such as punicic acid have been shown to be more cytotoxic to leukemia cells than dienoic conjugated fatty acids [21] such as those occurring in bovine products and known as "conjugated linoleic acid" (CLA). Punicic acid is also a potent inhibitor of prostaglandin biosynthesis from arachidonic acid [22].

That combining extracts from pomegranate juice and pomegranate peels might exert additive or supra-additive effects in cell culture was hinted at by studies with laboratory-obtained, versus commercial, pomegranate juice. Laboratory-obtained juice was extracted by pressing the arils (the juice-encapsulated seeds), which had been manually separated from the peels prior to pressing. Commercial pomegranate juice, on the other hand, is obtained by pressing whole fruits. The commercial juice, which is thus tainted from pressing the peels, is a stronger antioxidant in an *in vitro*, cell-free, chemical system [13], presumably because of inclusion of the pericarp tannins.

We previously reported complementary anti-cancer properties from pomegranate fermented juice polyphenols (anti-proliferative, anti-carcinogenic) and pomegranate seed oil (anti-invasive, pro-apoptotic) *in vitro* [19], as well as chemopreventive effects in breast *ex vivo* [16, 19], in skin *in vivo* [23] and in prostate *in vitro* and *in vivo* [24], and also antiangiogenic effects *in vitro* and *in vivo* [25]. The present work goes further in showing the effect of *combining* these fractions in various settings with human prostate cancer cells in culture. These include assays with DU 145 cells to assess proliferation, and an assay with the more aggressive PC-3 cells to assess invasion across Matrigel, an *in vitro* model of metastasis. Expression of the enzyme secretory

phospholipase A2 (PLA2) was also assessed as PLA2 has been specifically correlated with prostate cancer cell invasiveness [26] in particular and prostate cancer tumors in general [27]. Enzyme PLA2 controls eicosanoid metabolism, the conversion of arachidonic acid into leukotrienes, thromboxanes and prostaglandins, "uphill" from lipoxygenase and the cyclooxygenases, and is a likely partial mechanism for some of the anticancer effects observed thus far from pomegranate fractions.

Materials and methods

Preparation of pomegranate extracts

Organically grown pomegranate fruits (Wonderful cultivar, Sde Eliahu, Israel, 1999) were processed according to the methods previously described [18, 19] to yield pomegranate fermented juice polyphenols (W), pomegranate peel polyphenols (P) and pomegranate seed oil (Oil). The oil was dissolved drop-wise in stirred, heated ethanol to give a stock solution of 8.25 μg/ml.

Inhibition of proliferation of DU 145 human prostate cancer cells by a variable dose of W and a fixed dose of Oil. DU 145 human prostate carcinoma cells (American Type Culture Collection (Rockville, Maryland, USA) were cultured according to Manufacturer's instructions in a humidified incubator at 37°C and 5% CO_2. Dose responses of W, Oil and their combination were assessed by measuring cellular ATP (Bioluminescent ATP Assay, LumiTech, Nottingham, UK) according to Manufacturer's instructions, with modification [28]. Briefly, cells were plated in 96 well culture plates with 2×10^3 cells per well (Fischer Scientific, Ltd., Loughborough, UK). When Oil was used, the dose was 16.5 μg/ml. When W was employed, doses were finely graded from 0 to 100 μg/ml. Cells were incubated with these treatments for 96 h with repeat dosing at 48 h, then 100 μl of nucleotide releasing reagent added. The cells were then left for 30 min at room temperature to extract the ATP, quantified by adding 20 μl ATP monitoring reagent (containing luciferin and luciferase), to a micro-plate luminometer (Berthold Detection Systems, Fischer Scientific Ltd.).

Inhibition of proliferation of DU 145 human prostate cancer cells by variable dose of W and fixed dose of P. Human DU 145 prostate carcinoma cells (HTB-81, American Type Culture Collection, Manassas, VA) were grown in Eagle's Minimum Essential Medium modified by American Type Culture Collection, and supplemented with 10% fetal bovine serum, 1.0 mM sodium pyruvate/l, 0.1 mM nonessential amino acids/l; 1.5 grams sodium bicarbonate/l and 80 mg gentamycin/l. Cultures were seeded in 0.1 ml medium with 1,000 cells/well in a 96 well plate, and incubated 24 hr at 37°C in humidified 5% CO_2. The medium was then decanted from each well and replaced with 0.1 ml fresh medium containing the test agents and incubated an additional 72 hr. Cell populations of all wells were determined with CellTiter 96® (Promega Corporation, Madison, WI) procedure. Photomicrographs of representative fields of mono-layers of DU 145 cells were made with a Nikon Coolpix 995 digital camera attached to a Nikon TS 100, inverted microscope.

Inhibition of invasion of PC-3 human prostate cancer cells by W, P, Oil and their combinations. Our *in vitro* invasion assay was previously described [29]. Matrigel™ (Becton, Dickenson and Company, Franklin Lakes, NJ) was used to coat the 6.5 mm diameter polycarbonate membranes (pore size 8 μm) in the chambers of a 24-transwell system (Corning Costar Transwell, Cambridge, MA) at 50 μg/membrane. Following gel rehydration, 5×10^4 prostate cancer cells were added to each well. Hepatocyte growth factor/scatter factor (Becton, Dickenson & Company, Franklin Lakes, NJ) was used at 40 ng/ml to induce invasion. The upper chamber contained W, P, Oil, or a combination at a fixed total concentration of 3 μg/ml. After 72 hr culture, invasive cells stuck to the lower surface were fixed and stained with crystal violet, and their number quantified under an inverted microscope and expressed as percentage of positive control.

Inhibition of secretory phospholipase A2 (sPLA2) production by PC-3 human prostate cancer cells by W, P, Oil and their combinations. PC-3 human prostate cancer cells were grown in Dulbecco's Modified Eagles's Medium supplemented with 10% fetal calf serum for 72 hours, then treated with W, P, Oil, or their combinations, for a fixed concentration of 3 μg/ml for 30 min. RNA was extracted from the cells using an RNA extraction kit (ABgene, Surrey, UK), and its concentration determined with an ultraviolet spectrophotometer. A total of 1 μg RNA from each treated cell was used to generate complementary deoxyribonucleic acid (cDNA) using a reverse transcription kit (AB gene). The level of sPLA2 transcripts from the thus prepared cDNA was determined using a real-time quantitative polymerase chain reaction, based on the Ampliflour™ technology [30]. In short, pairs of polymerase chain reaction primers were similarly designed using Beacon Designer Software, Version 2 (PREMIER Biosoft International, Palo Alto, CA). However, to one of the primers was added the additional Z sequence (5′ actgaacctgaccgtaca ′3), complementary to the universal Z probe (Intergen, Inc., Sunnyvale, CA), i.e., 5′caggaactcacagagtggat′3 and 5′actgaacctgaccgtacagctctcatagaagggtagca′3. A Taqman detection kit for –actin (Perkin-Elmer, Wellesley, MA) was employed. The reaction was carried out using

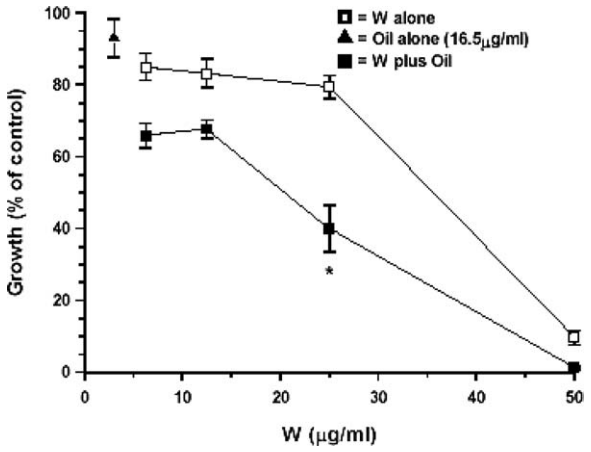

Figure 2. Synergism between pomegranate seed oil (Oil) and pomegranate wine polyphenols (W) in inhibiting proliferation of DU 145 human prostate cancer cells. A fixed dose of Oil (16.5 μg/ml) alone had no antiproliferative effect. The antiproliferative effect of W alone was dose dependent. A supra-additive effect was noted when Oil and W were combined, which peaked at 25 μg/ml W ($p < 0.001$).

Hot-start Q-master mix (Abgene), 10 pM specific forward primer, 1 pM reverse primer when containing the Z sequence, 10 pM of alkynyl 6-carboxyflourescein—tagged probe (Intergen) and cDNA from approximately 50 ng RNA. The IcyclerIQtm (Bio-Rad, Hercules, CA), equipped with an optical unit, allowed real time detection of 96 reactions with the following set of conditions: 94° for 12 min, 50 cycles of 94°C for 15 sec, 55°C for 40 sec and 72°C for 20 sec. The levels of transcripts were generated from a standard simultaneously amplified with the samples.

Statistical analysis. We analyzed all data with the Kruskal-Wallis non-parametric H test. We performed an additional median effect analysis [31] with CompuSyn software (Biosoft, Cambridge, UK) for the proliferation assays.

Results

Figure 2 depicts the effect on DU 145 prostate cancer cell proliferation of treatment with a fixed, sub-suppressive dose of Oil (16.5 μg/ml) and a range of non-suppressive and suppressive doses of W (0–100 μg/ml). Though Oil (16.5 μg/ml) alone does not affect proliferation, it synergistically enhances the antiproliferative effect of W, reaching a peak enhancement at 25 μg/ml W ($p < 0.001$). CalcuSyn median effect analysis produced a full range of points <1, confirming synergy (data not shown).

Figure 3a shows the antiproliferative effects on DU 145 prostate cancer cells of 6.25 μg/ml and 12 μ/g ml of pomegranate peel (pericarp) polyphenols P on increasing doses of W. At 6.25 μg/ml P has no effect on proliferation, but synergistically enhances the effect of W at a peak enhancement at 50 μg/ml W ($p < 0.001$). The same effect can be seen in the photomicrograph of Figure 3b. CalcuSyn median effect analysis produced a full range of points <1, confirming synergy (data not shown).

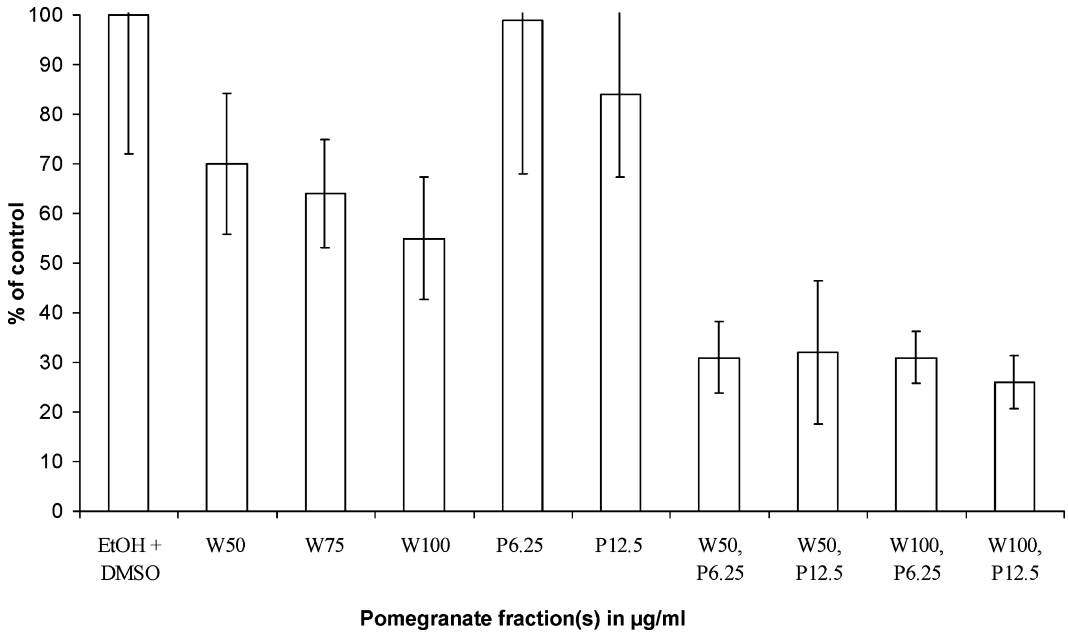

Figure 3a. Synergism between pomegranate wine polyphenols (W) and pericarp polyphenols (P) in inhibiting growth of DU 145 human prostate cancer cells. Maximum inhibition occurs at maximum dose of each used, synergy is observed at sub-effectual dose (6.25 μg/ml) of pericarp polyphenols and 25 μg/ml of fermented juice polyphenols ($p < 0.001$).

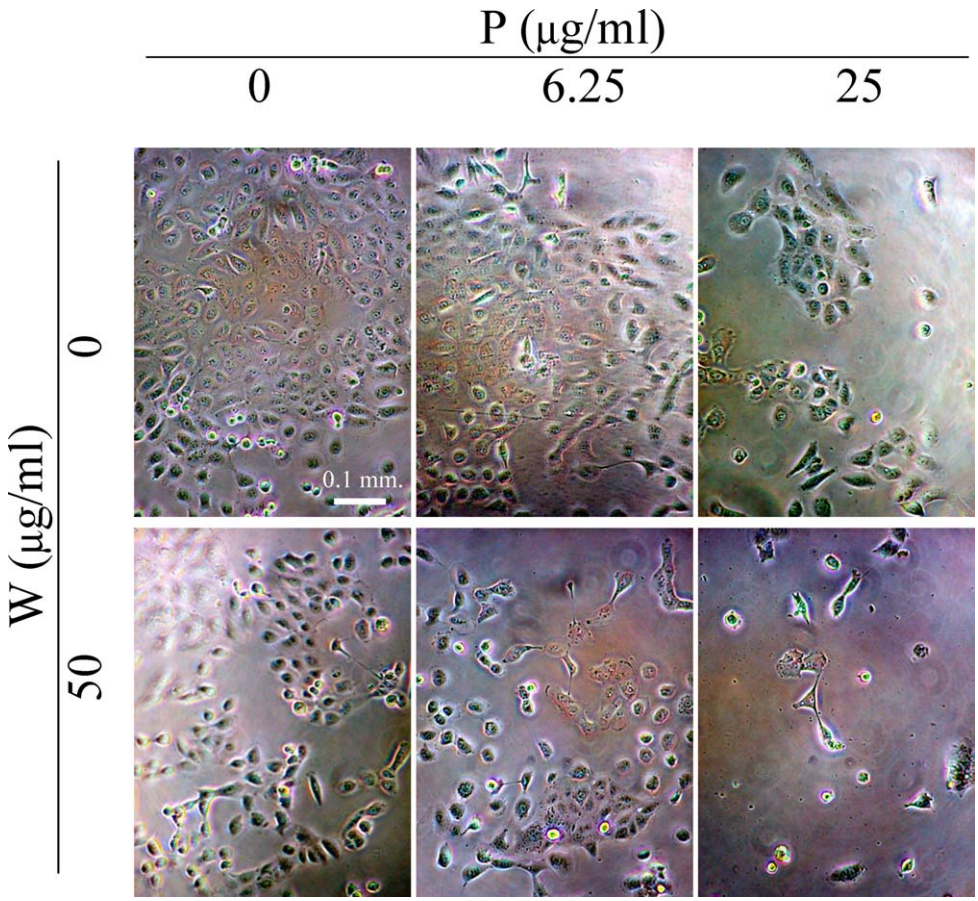

Figure 3b. Photomicrographs of DU 145 cells after a 72-hour incubation with pomegranate wine polyphenols (W) and pericarp polyphenols (P), individually and in combination. Inhibition by either pomegranate wine polyphenols or pomegranate pericarp polyphenols alone is dose-dependent, but synergy is observed between a sub-effectual dose of pomegranate pericarp polyphenols (6.25 μg/ml) and an active dose of pomegranate wine polyphenols (25 μg/ml).

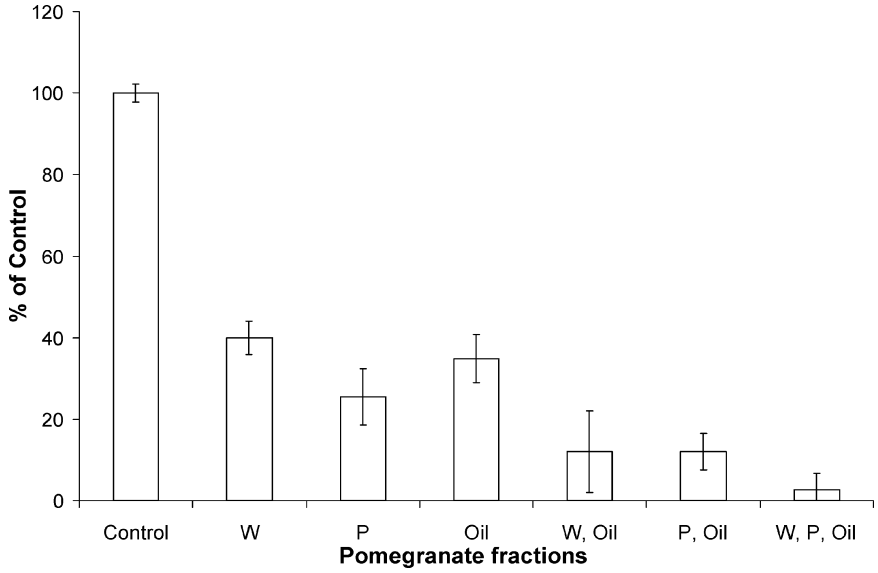

Figure 4. Synergism of pomegranate seed oil (Oil), pomegranate wine polyphenols (W), and pomegranate pericarp (peel) polyphenols (P) in suppressing invasion of PC-3 human prostate cancer cells through a Matrigel^{TM} membrane ($p < 0.01$).

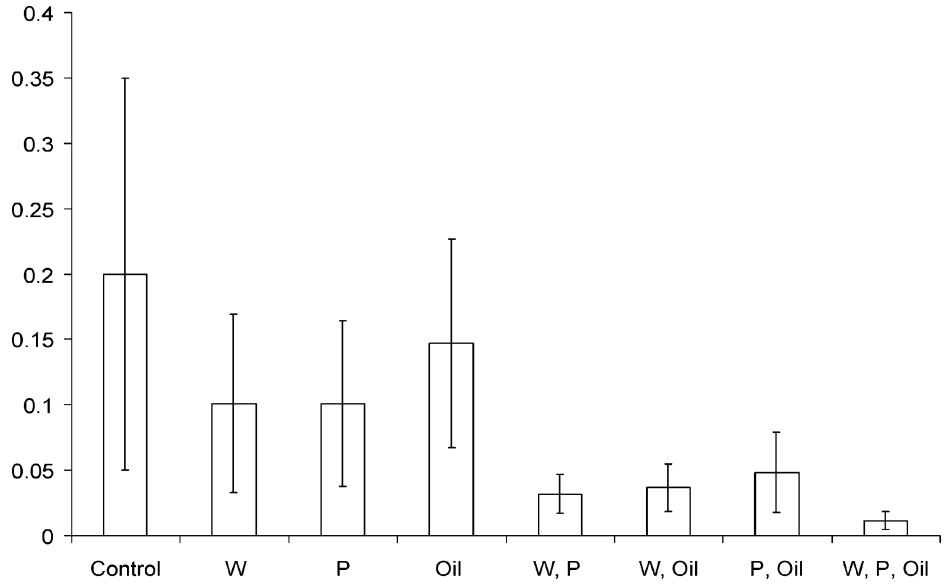

Figure 5. Supra-additive effects of combining pomegranate seed oil (Oil), pomegranate wine polyphenols (W) and pomegranate pericarp (peel) polyphenols on suppression of secretory phospholipase A2 (sPLA-2) measured as RNA expression from human PC-3 human prostate cancer cells. PLA-2 is uphill from cyclooxygenase and lipoxygenase in the metabolism of arachidonic acid and is an important component in cancerous tumors of the prostate. The wide error bars reflect the inherent variability of this assay, but the results here are significant ($p < 0.05$).

The synergistic effects of combining equal amounts of W, P and Oil are shown in Figure 4. Oil or W alone suppress invasion of PC-3 human prostate cancer cells across Matrigel^{TM} by about 60%, P by about 70%. When any two of these fractions are equally combined such that the total concentration remains 3 μg/ml, the effect on invasion approaches 90%, and when all three are combined equally for a total concentration of 3 μg/ml, the suppression of invasion approaches 99% ($p < 0.01$ in all cases).

Figure 5 shows the effect of combining the same fractions as for invasion on the expression of enzyme phospholipase A2 (PLA2) from PC-3 human prostate cancer cells. Oil alone resulted in approximately 25% reduction and W or P in 50% reduction. Combinations of any two of the three fractions effected 75–80% reductions, and all three together in about 95% reduction ($p < 0.05$ in all cases).

Conclusions

The preceding study clearly shows what appears to be synergy in the interactions of the extracts from the three pomegranate compartments (peels, juice and seeds) in inhibiting prostate cancer cell proliferation, invasion and phospholipase A-2 expression. The apparent synergistic interaction was robust and significant, but the question begs to be asked: was this truly synergy, or only a dose response, albeit a non-linear one, of a single compound present in all three fractions?

As reviewed previously, there is a good deal of overlap between the chemistry of the peels and the chemistry of the juice. The peel also contains "pro-drug" versions (pro-delphinidins) of compounds otherwise distinct to the juice (delphinidins). For this reason, although there is apparent synergy between fractions from the peel and the juice, it cannot be said with a high level of certainty that this is not due to compounds occurring in both fractions, exerting non-linear, pure molecule, dose responses. Nonetheless, the data leave no question, that, for whatever reason, the combination of comparable ethyl acetate extracts from the aqueous pericarp extract and the fermented juice concentrate of the pomegranate, do behave synergistically in a rigorous, mathematically verifiable manner, in inhibiting prostate cancer cell proliferation.

The case is quite different for seed oil versus fermented juice. Here, only very tiny amounts of oil were used, which by themselves were quite inactive. Polyphenols are contained in the oil, but only in extremely low concentrations, compared to much higher concentrations ($>50\times$) in the ethyl acetate extracts of fermented juice or peels. Thus, the chance of the observed synergy being due to a single compound acting from both the polyphenol fraction of the oil and the polyphenols contained in the ethyl acetate extracts of fermented juice or peels, is considerably more remote than in considering synergy between peels and juice. Certainly, the compounds which comprise the bulk of the seed oil (>99%) are not contained in either the peels or the juice.

We plan further studies with pure compounds to strengthen the hypothesis that the phenomena described herein are in fact due to synergistic interactions between different molecules. For example, a single compound found only in the oil (e.g., punicic acid) could be combined with a single compound from peels or juice that is definitely not found in the oil (e.g., ellagic acid). If synergy were observed between these compounds, it would reinforce the possibility that synergy is also present when the complex pomegranate fractions are combined.

Assuming that anti-cancer synergy does exist among different chemicals from a plant necessitates a radical overhauling of conventional wisdom in designing plant-based therapeutic agents. For if a therapeutic advantage can be obtained from mixing compounds rather than simply from further purifying and increasing the dose of the most active compound, then combinations of actives, rather than single actives, begin to look like the best strategic choices for fighting disease. Pharmacognocists might develop vertical (single plant) as well as horizontal (many plant) strategies for mining the botanical kingdom for new drugs.

Developing complex mixtures into *bona fide* "investigational new drugs" presents considerable tactical challenges. Simple quantification of single agents fails as a means of standardizing such a preparation, though recognizing key actives and measuring their relative proportions does provide a skeletal kind of order. A further step, however, can come from capturing information about the complex mixture as a whole, such as is obtainable with high powered nuclear magnetic resonance (NMR) imaging of the entire molecular landscape and the subsequent bio-informatic analysis of the resulting data sets (i.e., metabolomics or metabonomics) [32, 33]. Ideally, standardization of a complex botanical drug comprising different pomegranate fractions could involve both quantification of key compounds (analogous to characterizing the particle nature of light) and also describing the overall molecular landscape with metabolomics (analogous to the wave nature of light).

Plants evolve complex biochemical networks for solving their own requirements, such as the use of steroid dehydrogenases for fixing nitrogen [34], or production of "secondary metabolites" for defense against insects [35] or inducing infertility in mammalian predators [36]. In spite of Nature's preference for parsimony, redundant and multiple pathways are frequently utilized, probably because they contribute to overall stability and resilience. Resistance of disease processes to pure chemical agents is frequently a problem in medicine, which has led to the strategy, as in Nature, of employing multiple agents for achieving specific goals: for treating hypertension, for enhancing cytotoxic potency [37, 38] against cancer cells, and for targeting secondary systems, such as the vascular system, thus increasing overall cancer chemotherapeutic efficacy [39]. Because single plants evolve complex means for fighting their own enemies, and because cancer clones also evolve complex mechanisms for attaining advantage over their hosts [40, 41], the chemical complexity used by plants for their own purposes may also be in part transferable to the drug developer for attacking medical problems, including cancer, in man [42].

Future explorations with the pomegranate will need to focus firstly on the comprehensive characterization of the active fractions and how they can be best combined for optimal effect. Initial pharmacokinetic and toxicological studies with some of the active components have shown both good absorption into the blood stream and a high profile of safety [43–45], and must be expanded to include the full therapeutic mixture(s) of components and specific tracking to the active site(s) desired (e.g., the prostate). Once these objectives have been achieved, the investigational new complex agent will be subjected to *in vivo* efficacy testing and ultimately, clinical investigations.

In short, the preceding study is a very early inquiry into how the pharmacological richness of the pomegranate fruit might lead to a complex, synergistic drug for the treatment of cancer. It provides another chapter not only to our own explorations [46], but also to those of others who have highlighted, for example, the fruit's potential for reducing and preventing atherosclerosis [47–49]. In that the pomegranate fruit has been recognized since antiquity not only as a medicinal article, but actually as a symbol for medicine itself [50], these investigations might thus also be regarded as timely.

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Address for offprints: Ephraim Philip Lansky, Scientific Director, Rimonest Ltd., Horev Center, Box 9945, Haifa, Israel. Fax: 972-4-834-0966; Cell: 972-545-273-156; Email: info@rimonest.com