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Pomegranate (*Punica granatum*) pure chemicals show possible synergistic inhibition of human PC-3 prostate cancer cell invasion across Matrigel™

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Summary

Four pure chemicals, ellagic acid (E), caffeic acid (C), luteolin (L) and punicic acid (P), all important components of the aqueous compartments or oily compartment of pomegranate fruit (*Punica granatum*), and each belonging to different representative chemical classes and showing known anticancer activities, were tested as potential inhibitors of *in vitro* invasion of human PC-3 prostate cancer cells in an assay employing Matrigel™ artificial membranes. All compounds significantly inhibited invasion when employed individually. When C, P, and L were equally combined at the same gross dosage (4 μg/ml) as when the compounds were tested individually, a supradditive inhibition of invasion was observed, measured by the Kruskal-Wallis non-parametric test.

The tannin, ellagic acid (E) [1], the phenolic acid, caffeic acid (C) [2], the estrogenic flavone, luteolin (L) [3] and the conjugated trienoic fatty acid, punicic acid (P) [4], are compounds with known anti-cancer actions found in substantial amounts in the peels, juice and seed oil of the pomegranate fruit (*Punica granatum*). Polyphenol-rich fractions of pomegranate fermented juice and peels, and pomegranate seed oil, when combined, supra-additively inhibit proliferation, invasion and secretory phospholipase A2 (sPLA2) expression in human prostate cancer cells [5]. To evaluate a possible contribution in this effect of these class-representative active compounds with likely different modes of action, the present study was launched.

The compounds were obtained commercially: E. C, L from Sigma Aldrich (Rehovot, Israel), P from Larodan Chemicals (Malmo, Sweden), and used in a double blind assay for *in vitro* invasion [6], in which hepatocyte growth factor/scatter factor (HGF) (Becton, Dickenson & Company, Franklin Lakes, NJ) was used to stimulate invasion of 5×10^4 human PC-3 prostate cancer cells added to each Matrigel™ (Becton, Dickenson and Company, Franklin Lakes, NJ) pre-coated chamber of a 24-transwell system (Corning Costar Transwell, Cambridge, MA). Chemicals were added in equal weights, commensurate with our previous experience, to the upper wells at 4 μg/ml , regardless of whether one, two, three or all four of the agents were employed.

The relative closeness of the molecular weights ($E = 302$, $C = 180$, $L = 286$, $P = 278$) allowed a general comparison of the compounds by gross weight. In that E, C and L are contained in high percentages in the peel/juice polyphenol fractions and punicic acid predominates in pomegranate seed oil, the concentrations used were deemed appropriate.

After 72 h, invasive cells stuck to the lower transwell surfaces were fixed and stained with crystal violet, their number quantified with inverted microscopy and expressed as a percentage of the positive control.

Ten separate assays tested the positive control (HGF only) and five for each experimental drug combination. The Kruskal-Wallis test was used to test for differences, with $p < 0.05$ considered significant. Paired comparisons between all five values from two different groups ($p = 0.03$) were used to increase sensitivity if only borderline significance by the Kruskal-Wallis test was observed.

Single agents individually inhibited invasion by $p \leq 0.0196$; combinations of any two compounds showed supra-additive, non-significant trends. Combination L, C, P was significantly better than any single agent (Table 1)

Table 1. Effects of pure chemicals on PC-3 Invasion

Single agents			Doublets			Triplets			Quadruplet		
	X	*s*		X	*s*		X	*s*		X	*s*
L	9	1.6	*LP*	5.2	2.6	*PCE*	9.0	3.4	*LPCE*	5.4	3.4
P	6.4	1.1	*LC*	6.2	4.0	*LP*	1.8	1.8			
C	16.8	3.4	*LE*	6.0	2.2	*CLP*	3.4	2.3			
E	7.4	2.3	*PC*	5.0	1.0	*E*					
c+	33	10.7	*PE*	4.2	2.0						
c−	3	1.7	*CE*	5.2	1.3						

L = luteolin, P = punicic acid, C = caffeic acid, E = ellagic acid, $c+$ = positive control, $c-$ = negative control, $\underline{X}$ = mean number of invading cells, and s = standard deviation.

and also better than P, L alone ($p = 0.03$ by paired comparison).

Although caution must be exercised in interpretation, the Kruskal-Wallis treatment of the results strongly suggests a supra-additive, possibly synergistic effect in inhibiting *in vitro* prostate cancer cell invasion when these chemicals are equally, or near-equally (owing to their different molarities) combined.

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