Breast cancer chemopreventive properties of pomegranate (*Punica granatum*) fruit extracts in a mouse mammary organ culture

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We previously reported anticancer effects of pomegranate extracts in human breast cancer cells *in vitro* and also chemopreventive activity of pomegranate fermented juice polyphenols (W) in a mouse mammary organ culture (MMOC). In the present study we decided to expand the MMOC investigations to also include an evaluation of the potential chemopreventive efficacy of a purified chromatographic peak of W (Peak B), and also of whole pomegranate seed oil. In brief, an MMOC was established according to a known method. For the first 10 days of culture, the glands were treated with pomegranate fermented juice polyphenols (W), a high-performance liquid chromatographic (HPLC) peak separated from W (peak B), or pomegranate seed oil (Oil, and on day 3, exposed to the carcinogen 7,12-dimethylbenz[a]anthracene (DMBA), and for 10 days treated with the putative pomegranate chemopreventive. The glands were subsequently harvested and tumours counted by visual inspection. While W effected a 42% reduction in the number of lesions compared with control, peak B and pomegranate seed oil each effected an 87% reduction. The results highlight enhanced breast cancer preventive potential both for the purified compound peak B and for pomegranate seed oil, both greater than that previously reported for pomegranate fermented juice polyphenols. *European Journal of Cancer Prevention* 13:345–348 © 2004 Lippincott Williams & Wilkins.

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Introduction

Pomegranate (*Punica granatum*) is an ancient fruit with extensive regard both as a symbol and as an article of medicine (Langley, 2000). Our previous work with pomegranate fractions demonstrated antioxidant and eicosanoid enzyme inhibition (Schubert *et al.*, 1999), suppression of breast cancer growth and invasion, and inhibition of breast cancer carcinogenesis (Kim *et al.*, 2002), inhibition of skin cancer carcinogenesis (Hora *et al.*, 2003), and inhibition of angiogenesis (Toi *et al.*, 2003). Additional pharmacological actions have also been recorded, especially that owing to both the prevention and reversal of atherosclerotic lesions (Aviram *et al.*, 2002).

In earlier studies we showed that the effects of pomegranate fruit may be largely due to the presence of selective polyphenols. However, the selective role of each polyphenol in mouse mammary organ culture (MMOC) has not been investigated. Previously we showed that the fruit extract (W) exhibits chemopreventive activity in MMOCs (Kim *et al.*, 2002). However, the effect was not dramatic. Here we intended to compare the activity of the fruit extract (W) with that of isolated high-performance liquid chromatography (HPLC) peaks in mammary lesion formation. We selected one of these isolates, designated as peak B and compared its activity with that of the parent fruit extract. Pomegranate seed oil is rich (~80%) in conjugated fatty acids and contains small amounts of other physiologically active compounds such as gamma-tocopherol, campestrol, sigmasterol, sitosterol, estrone and alpha-estradiol (Table 1). Punicic acid alone, which comprises 65% of the whole oil, is a significant inhibitor of prostaglandin biosynthesis (Nugteren and Christ-Hazelhof, 1987). Thus pomegranate seed oil, which we found to be a potent inhibitor of breast cancer cell invasion in very small doses (3 μg/ml) (Kim *et al.*, 2002) may also have broader potential as an anti-inflammatory agent.

We compared here the activity of pomegranate seed oil with that of the fermented juice extract (W) and HPLC isolate (peak B). The present study is the first to examine the potential chemopreventive utility of a possibly novel pomegranate compound (peak B), and in addition, expands the known potential chemopreventive applications for pomegranate seed oil.

Materials and methods

Pomegranate extracts

Organically grown pomegranates of the 'Wonderful' cultivar from the 2000 crop of Kibbutz Sde Eliahu, Israel

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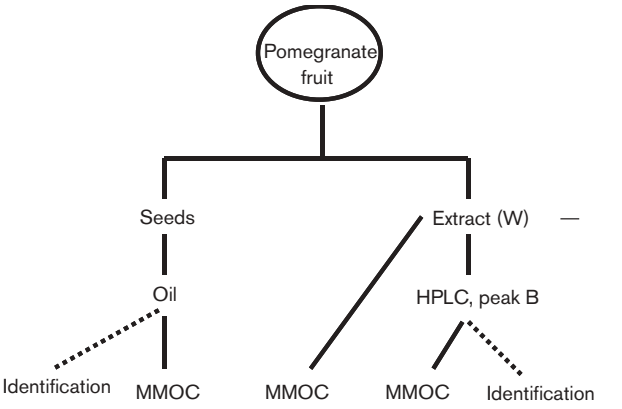
Table 1 Analysis of pomegranate seed oil used in this study

	%	mg/g
Fatty acid profile		
16:0 palmitic	3.6	9.6
18:0 stearic	2.2	6.0
18:1 oleic	6.8	18.5
18:2 linoleic	6.5	17.4
18:3 γ-linolenic	0.6	1.2
18:3 conjugated trienes	65.3	175.6
20:0 icosanoic	0.4	1.1
20:1 icosenoic	0.6	1.7
22:0 docosanoic	1.3	3.4
22:1 docosenoic	2.9	8.0
22:5 docosapentaenoic	0.3	1.1
24:0 tetracosanoic	1.4	3.0
24:1 tetracosenoic	0.5	1.1
Minor components[a]	7.6	
C18 and C46 species		
Phospholipid	3	
Monoacylglycerol	1	
Diacylglycerol	8	
Triacylglycerol	80	
Wax esters	7	

[a]Minor components were: sitosterol (0.6%), campesterol (0.1%), stigmasterol (0.01%), gamma-tocopherol (0.3%), 17 alpha estradiol (0.3%) and other (~1.5%).

Fig. 1



Extraction procedure for identifying and evaluating pomegranate extracts and isolates.

were utilized to produce all the putative chemopreventive agents. Pomegranate seed oil was prepared by cold press at 80°C using an electric seed pressing machine (40A; Skeppsta Maskin, Orebro, Sweden) from washed and dried seeds from which the juice had been previously removed. Pomegranate fermented juice polyphenols (W) were obtained by layering concentrated fermented pomegranate juice over ethyl acetate overnight (12 h). In the morning, the ethyl acetate layer was separated with a separation funnel, and evaporated under nitrogen gas to yield the polyphenols (W). Peak B was obtained from a specific peak on a high-performance liquid chromatogram using preparative HPLC technique. The extraction is summarized in Figure 1.

Mouse mammary organ culture

A murine mammary gland organ culture was established according to a known method (Mehta *et al.*, 1991, Mehta 2000). The study was approved by the University of Illinois Animal Review Board and performed in accordance with institutional guidelines. Briefly, young, virgin BALB/c female mice, 3–4 weeks of age (Charles River Laboratories, Wilmington, Massachusetts, USA) were pre-treated for 9 days with 17β-estradiol (1 μg in 0.1 ml of saline/animal) and progesterone (1 mg in 0.1 ml of saline/animal). They were subsequently killed by cervical dislocation, and the thoracic pair of mammary glands removed, placed on silk rafts, and incubated for 10 days in serum-free Waymouth MB752 medium (Life Technologics, Inc, Gaithersburg, Maryland, USA) containing the following growth-promoting hormones: insulin (5 μg/ml), prolactin (5 μg/ml), aldosterone (1 μg/ml) and hydrocortisone (1 μg/ml). The carcinogen 7,12-dimethylbenz-[a]anthracene (DMBA) at a dose of *2* μg/ml in DMSO was added to the medium on day 3 for a duration of 24 h to induce mammary lesions.

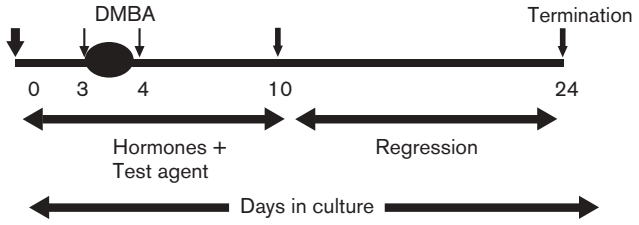
The mammary glands were incubated for an additional 14 days with medium containing only insulin. This procedure allowed the normal glands to undergo structural regression in which all the normal alveolar structures were disintegrated. But alveolar lesions in the carcinogen-treated glands behave differently, namely, they acquire altered hormone responsiveness and continue to grow.

Between 11–15 mammary glands per group were employed in four different groups: a control, and those treated with either fermented juice polyphenols (W), purified fraction peak B, or pomegranate seed oil (oil). The three agents were individually included in the media during the first 10 days of *in vitro* culture to determine if they lowered the incidence of formation of mammary lesions. Throughout the culture period, the glands were maintained at 37°C in a 95% O_2 and 5% CO_2 atmosphere. At the end of the culture period, the glands were fixed in formalin, stained in alum-carmine solution, and evaluated for presence or absence of mammary lesions. The multiplicity of the lesions (number of lesions per gland) was not scored for the present study. Previous studies from our laboratory have shown that in using 15 glands per group the incidence varies from 53 to 100% of glands with lesions. All hormones and chemicals were purchased from Sigma Chemical Company (St Louis, Missouri, USA). The procedure is shown in Figure 2.

Statistical analysis

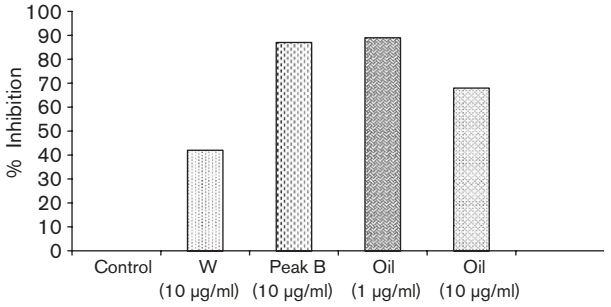
Probabilities for the relative differences between the experimental groups were determined with the

Fig. 2



Experimental design to induce mammary lesions in the gland – its prevention by pomegranate isolates.

Fig. 3



Effect of different pomegranate fractions on suppression of tumorigenesis in a mouse mammary organ culture model. W = pomegranate fermented juice polyphenols; peak B = semi-purified peak separated from W by preparative high-performance liquid chromatography; oil = cold-pressed pomegranate seed oil. Significance: $P < 0.05$ in all cases by chi-square analysis. $N = 11–15$ glands per group.

chi-square test, with $P < 0.05$ considered significant. In general, assuming 60% incidence of lesions in control glands, and 60% suppression by a chemopreventive agent in relation to control glands results in statistically significant inhibition using chi-square analysis.

Results

Previous studies with MMOC have shown that treatment with DMBA (2 µg/ml medium) induces precancerous mammary lesions in the gland with a frequency of 60–100%. Consistent with these results, in the present experiment 73% (11 of 15) of the control glands had mammary lesions. The phenolic extract of the fermented fruits (W) showed mammary lesion incidence of 37% (six of 13 glands with lesions) resulting in 38% inhibition of mammary lesions formation. However the effect was not statistically significant. On the other hand, treatment with both pomegranate seed oil and the purified HPLC peak (peak B) derived from a phenolic fraction of fermented pomegranate juice (W) demonstrated significantly greater chemopreventive potential than W. As compared with 38% inhibition of lesion formation by W, there was 75–90% suppression by peak B and seed oil ($P < 0.05$). The chemopreventive potential of the seed oil was greater at low dose (1 µg/ml) than normal dose (10 µg/ml). The experiment was repeated and the same result obtained. These results are shown in Figure 3.

Discussion

The MMOC provides at least a 75% predictive accuracy of *in vivo* carcinogenesis (Mehta, 2000), here strongly suggesting a chemopreventive role for pomegranate fractions in breast cancer. The success of the peak B in this model demonstrates the existence of a fraction possibly representing a pure compound within the fermented juice phenolic mixture possessing greater chemopreventive utility than the parent extract. Efforts to further purify and identify this compound are in progress.

Especially striking is the chemopreventive activity of the pomegranate seed oil, which appears to peak at very low doses. In this study, at least, this oil demonstrated chemopreventive activity equivalent to that of the semi-purified compound from the juice fraction. Pomegranate oil consists largely of punicic acid, a conjugated 18-carbon trienoic acid. The punicic acid and its isomers constitute approximately 80% of the oil (Schubert *et al.*, 1999). Trienoic acids such as punicic acid exert cancer suppressive utility greater than that well established for dienoic acids, such as the bovine-derived conjugated linoleic acid (CLA) (Igarashi and Miyazawa, 2000). The chemopreventive effect of pomegranate seed oil likely owes much to punicic acid, though inhibition of eicosanoid metabolism leading to prostaglandin biosynthesis has also been demonstrated for the polyphenol component ($< 1\%$) of the oil alone.

This preliminary report clearly suggests that both the pomegranate seed oil and fermented fruit extracts exhibit chemopreventive activity and the activity of the fruit extract may in part be due to the phenolic compound present in peak B of the HPLC isolate, whereas the efficacy of seed oil may be due to the presence of punicic acid present in the oil. Challenges lie ahead to design chemopreventive protocols to get the most out of the potential inherent in pomegranate. Pomegranate juice alone, though helpful, obviously misses the great potential of pomegranate seed oil. A clue to designing effective pomegranate chemopreventives might be taken from the painting *Proserpina* by the nineteenth-century Italian painter Dante Gabriel Rossetti, where the mystery maiden looks back at us having apparently

just taken a bite out of a whole pomegranate, rind, seeds and all (www.sisterzeus.com)! While pursuit of pure active compounds is the traditional occupation of drug discovery, the best strategy might yet be one which makes use of different parts of the fruit, all of which have demonstrated chemopreventive activity concealed within.

Acknowledgements

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