

## Differentiation-Promoting Activity of Pomegranate (*Punica granatum*) Fruit Extracts in HL-60 Human Promyelocytic Leukemia Cells

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**ABSTRACT** Differentiation refers to the ability of cancer cells to revert to their normal counterparts, and its induction represents an important noncytotoxic therapy for leukemia, and also breast, prostate, and other solid malignancies. Flavonoids are a group of differentiation-inducing chemicals with a potentially lower toxicology profile than retinoids. Flavonoid-rich polyphenol fractions from the pomegranate (*Punica granatum*) fruit exert anti-proliferative, anti-invasive, anti-eicosanoid, and pro-apoptotic actions in breast and prostate cancer cells and anti-angiogenic activities *in vitro* and *in vivo*. Here we tested flavonoid-rich fractions from fresh (J) and fermented (W) pomegranate juice and from an aqueous extraction of pomegranate pericarps (P) as potential differentiation-promoting agents of human HL-60 promyelocytic leukemia cells. Four assays were used to assess differentiation: nitro blue tetrazolium reducing activity, nonspecific esterase activity, specific esterase activity, and phagocytic activity. In addition, the effect of these extracts on HL-60 proliferation was evaluated. Extracts W and P were strong promoters of differentiation in all settings, with extract J showing only a relatively mild differentiation-promoting effect. The extracts had proportional inhibitory effects on HL-60 cell proliferation. The results highlight an important, previously unknown, mechanism of the cancer preventive and suppressive potential of pomegranate fermented juice and pericarp extracts.

**KEY WORDS:** • breast cancer • chemoprevention • flavonoid • prostate cancer

### INTRODUCTION

**D**IFFERENTIATION, the transformation of a cell toward its particular specialized function, is an important approach to cancer therapy.<sup>1–3</sup> Although differentiation therapy (and/or chemoprevention) is best known for hematological malignancies, particularly leukemia,<sup>4,5</sup> differentiation-based treatments are also applicable to prostate,<sup>6–8</sup> breast,<sup>9,10</sup> and other<sup>11</sup> cancers as well.

Probably the most important pro-differentiation agents for cancer therapy are the retinoids (*i.e.*, vitamin A derivatives).<sup>12,13</sup> Retinoids act at retinoid receptors, part of the steroid/thyroid superfamily of receptors.<sup>14</sup> Although retinoids are not without toxicity,<sup>15,16</sup> in general the path of differentiation can often be viewed as preferable, if not complementary, to chemotoxic treatments.<sup>17</sup>

Flavonoids represent a potentially less toxic path to differentiation than retinoids.<sup>18</sup> Flavonoids are generally plant-derived compounds, which often exert steroid-like effects, owing to their similar chemical structures.<sup>19</sup> Flavonoids also share certain mechanisms of action with retinoids,<sup>20,21</sup> sug-

gesting possible joint use to enhance the overall pro-differentiation effect. Specific flavonoid groups that promote differentiation derive from milk thistle (the complex is known as silymarin),<sup>22–24</sup> *Ginkgo biloba*,<sup>25</sup> curcumin,<sup>26</sup> and citrus.<sup>27,28</sup> Individual naturally occurring plant flavonoids that promote differentiation include quercetin,<sup>29</sup> luteolin,<sup>30</sup> and kaempferol.<sup>31</sup> Further, the first clinical trial of a cyclin-dependent kinase inhibitor/differentiating agent for cancer was conducted with a synthetic flavonoid.<sup>32</sup>

We have established the presence in pomegranate of several flavonoids possessing estrogenic properties. We have previously reported on kaempferol, quercetin, and luteolin in pomegranate peels.<sup>33</sup>

We have also previously assessed flavonoid-rich, pomegranate phenolic fractions for anti-cancer activity. Thus far, we have described anti-proliferative, anti-invasive, and pro-apoptotic effects against breast and prostate cancer cells *in vitro* (M. Albrecht *et al.*, manuscript submitted for publication), chemopreventive effects in murine breast *ex vivo*<sup>34</sup> and murine skin *in vivo*,<sup>35</sup> and a suppressive effect on human prostate cancer xenograft growth *in vivo* (M. Albrecht *et al.*, manuscript submitted for publication). Further, we have demonstrated inhibition of secretion of angiogenic growth factors (particularly vascular endothelial growth factor) and other *in vitro* indices of angiogenesis, as well as inhibition of angiogenesis in chick chorioamniotic membrane *in vivo*.<sup>36</sup>

The present work sought to assess the potential of these flavonoid-rich pomegranate extracts to affect differentiation in HL-60 human leukemia cells. By virtue of these cells' ability to differentiate into non-dividing phagocytes, this is an appropriate classic cell line for studying differentiation<sup>37,38</sup> pertinent to the putative therapy of leukemia and for cancer in general.

## MATERIALS AND METHODS

### Preparation of pomegranate flavonoid-rich fractions

Pomegranates used were of the 1999 crop of Sde Eliahu Israel. The fruit was juiced, and fermented juice polyphenols (fraction W)<sup>39</sup> and pericarp polyphenols (fraction P)<sup>34</sup> were prepared as previously described. The fresh pomegranate juice polyphenols (fraction J) were prepared in a similar manner. Specifically, the fresh juice was first concentrated over an open fire to about 50% solids, and this concentrate was layered over ethyl acetate for 12 hours. The ethyl acetate layer was then separated, and the ethyl acetate was removed with N<sub>2</sub> gas to yield J.

### HL-60 cell culture and treatments

HL-60 cells, obtained from the Riken Gene Bank (Tsukuba, Japan), were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum (Bio Whittaker, Inc., Walkersville, MD). HL-60 cells in log phase ( $\sim 10^6$  cells/mL) were diluted to  $1.2 \times 10^5$  cells/mL and preincubated for 18 hours in 24-well plates ( $\sim 2 \times 10^5$  cells/mL). Pomegranate fractions dissolved in dimethyl sulfoxide were then added, keeping the final dimethyl sulfoxide concentrations lower than 0.1% (vol/vol). After 4 days of incubation, the cells were analyzed to determine the percentage exhibiting morphological and biochemical differentiation. (In a blank experiment, differentiation of HL-60 cells was not induced in the presence of 0.1% dimethyl sulfoxide.)

### Nitro blue tetrazolium (NBT) reducing activity reducing assay

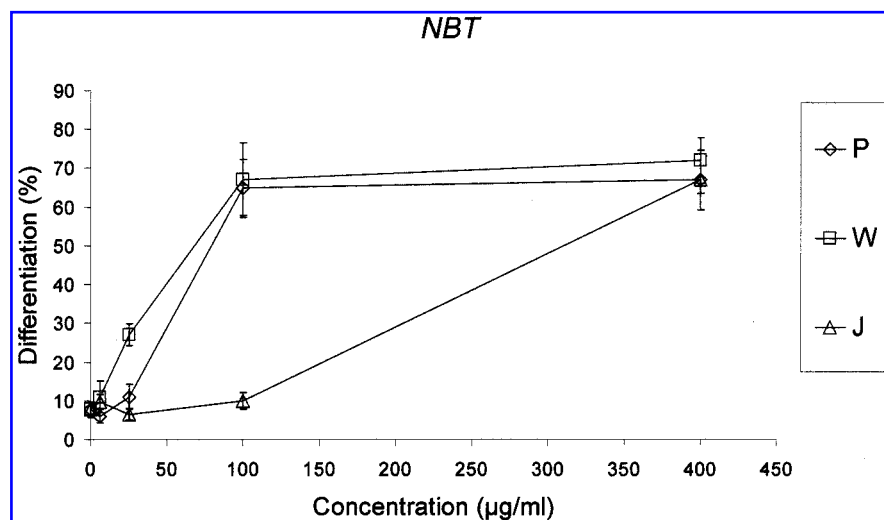
A 1:1 (vol/vol) mixture of a cell suspension ( $10^6$  cells/500  $\mu$ L) and freshly prepared 12-*O*-tetradecanoylphorbol 13-acetate (TPA)/NBT solution (phosphate-buffered saline solution containing 1 mg/mL NBT and 1  $\mu$ g/mL TPA) was incubated for 15 minutes at 37°C. Cells were then smeared on slide glass, and counterstained by 0.25% (wt/vol) safranin O in 10% ethanol. Differentiated cells, which gave an intracellular black-blue formazan deposit, were examined by counting a minimum of 200 cells in triplicate for each experiment.

### Nonspecific esterase (NSE) activity and specific esterase (SE) activity

Assays by  $\alpha$ -naphthyl acetate esterase (nonspecific acid esterase, NSE) and by naphthyl AS-D chloracetate esterase (specific acid esterase, SE) were done using cytochemical kits (91-A and 91-C, respectively) from Sigma Chemical Co. (St. Louis, MO). Differentiated cells were examined by counting a minimum of 200 cells in triplicates for each experiment.

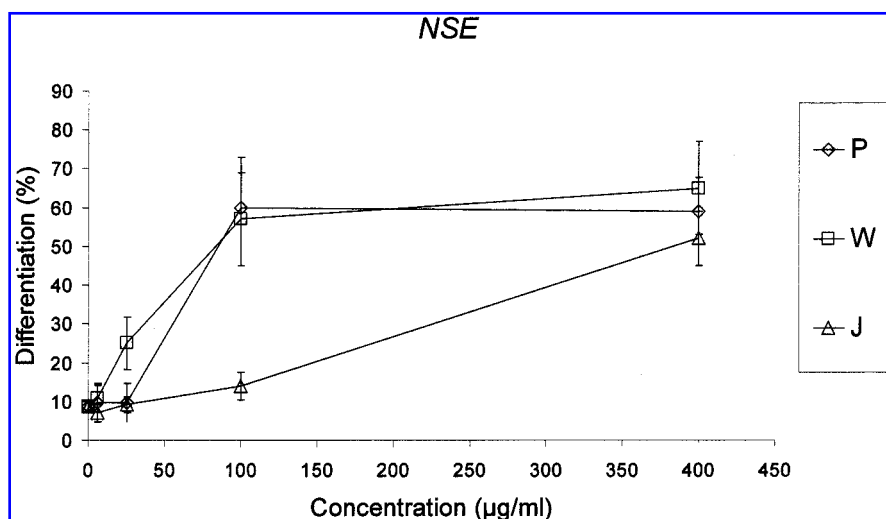
### Phagocytic activity

Polystyrene latex particles (average diameter, 0.81  $\mu$ m; Difco Laboratory, Detroit, MI) were suspended in RPMI 1640 medium at a concentration of  $10^9$  particles/mL. Pomegranate-treated HL-60 cells were washed and suspended in RPMI 1640 medium containing 20% AB serum (Bio Whittaker) at a final concentration of  $2 \times 10^6$  cells/mL. A 1:1 mixture of the latex particles and HL-60 cell suspensions was incubated for 4 hours at 37°C. Phagocytic activity was determined by counting cells ingesting the latex particles with a hemacytometer on a minimum of 200 cells.



**FIG. 1.** Effect of pomegranate fermented juice polyphenols (fraction W), pomegranate pericarp (peel) polyphenols (fraction P), and pomegranate fresh juice polyphenols (fraction J) on differentiation of HL-60 human leukemia cells according to the NBT reducing activity assay.

**FIG. 2.** Effect of pomegranate fermented juice polyphenols (fraction W), pomegranate pericarp (peel) polyphenols (fraction P), and pomegranate fresh juice polyphenols (fraction J) on differentiation of HL-60 human leukemia cells according to the NSE activity assay.



### Cell proliferation assay

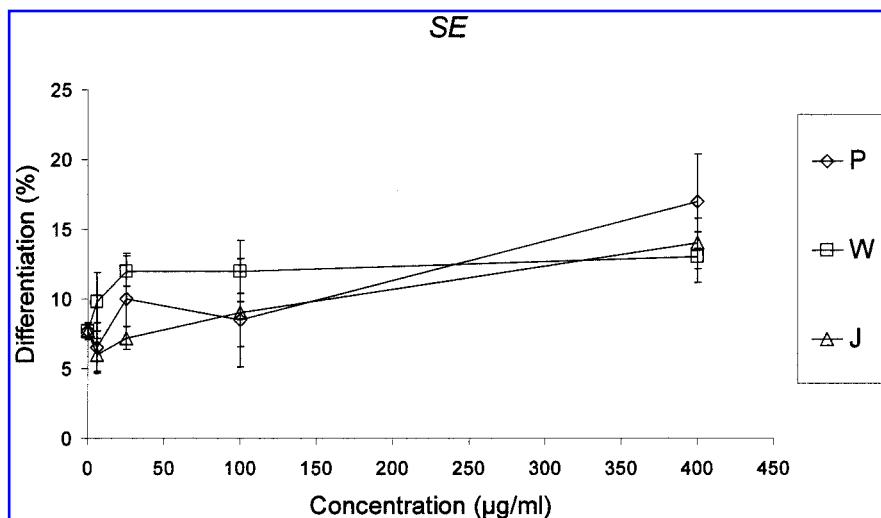
The level of cell proliferation was measured by using alamar Blue™ (Biosource International, Lewisville, TX), an oxidation–reduction indicator. The level of proliferation was measured for HL-60 cells grown in 96-well microtiter plates. Triplicate plates were prepared. To each well  $5 \times 10^3$  cells/100 µL of HL-60 cell suspension was added, grown for 24 hours, and then mixed with 100 µL of medium containing serial dilutions of fraction P, W, or J. Usually 8 µL of the dimethyl sulfoxide solution to be assayed was added to 1 mL of medium, and fourfold serial dilution was done in the microtiter plates so that the final volume in each well was 200 µL, and the final dimethyl sulfoxide concentrations did not exceed 0.4% (vol/vol). After 3 days of incubation, 20 µL of alamar Blue was aseptically added to each well, and incubated for 6 or 24 hours. Cellular proliferation (per-

centage of untreated control) was calculated with the following equation:

$$\text{Proliferation} = 100 \times \frac{[(A_{750} - A_{595}) \text{ of test agent dilution}] - [(A_{750} - A_{595}) \text{ of blank}]}{[(A_{750} - A_{595}) \text{ of untreated positive growth control}] - [(A_{750} - A_{595}) \text{ of blank}]}$$

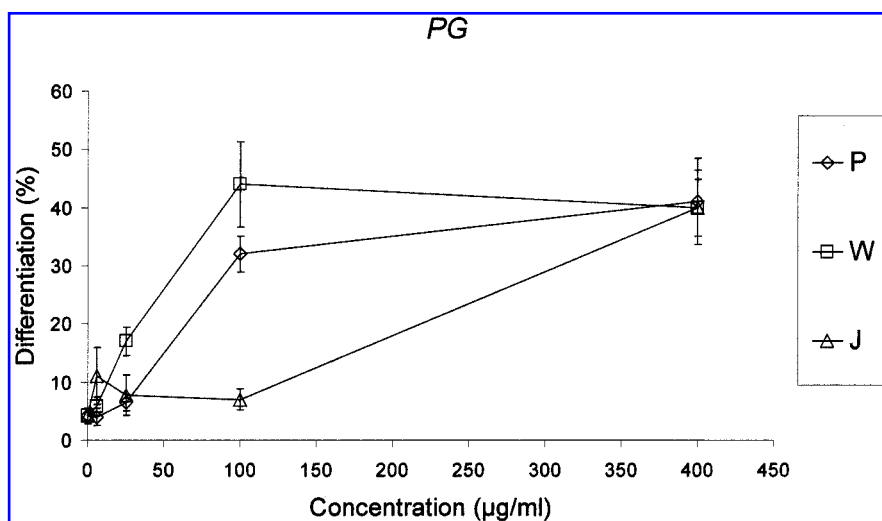
### RESULTS

Figures 1–4 show the effect of fractions W, P, and J on differentiation of HL-60 human promyelocytic leukemia cells according to NBT reducing activity, NSE activity, SE activity, and phagocytic activity assays, respectively. As can be readily noted, fractions W and P promoted differentia-



**FIG. 3.** Effect of pomegranate fermented juice polyphenols (fraction W), pomegranate pericarp (peel) polyphenols (fraction P), and pomegranate fresh juice polyphenols (fraction J) on differentiation of HL-60 human leukemia cells according to the SE activity assay.

**FIG. 4.** Effect of pomegranate fermented juice polyphenols (fraction W), pomegranate pericarp (peel) polyphenols (fraction P), and pomegranate fresh juice polyphenols (fraction J) on differentiation of HL-60 human leukemia cells according to the phagocytic (PG) activity assay.



tion about equally and strongly. The effect of fraction J on differentiation was considerably milder.

Figure 5 depicts the effect of fractions W, P, and J on proliferation of HL-60 cells. Here the effect is roughly a mirror image of the differentiation assays. Specifically, fractions W and P inhibit proliferation about equally and strongly, with a milder effect shown for fraction J.

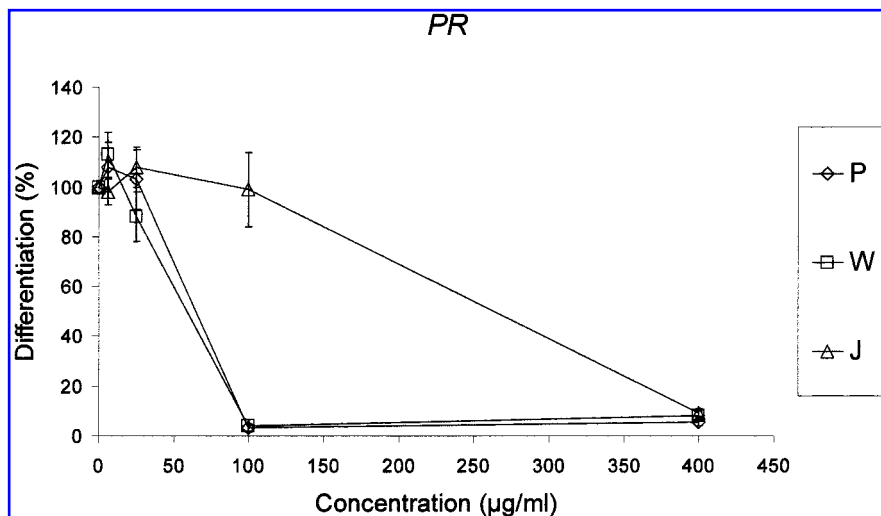
## CONCLUSIONS

The results show a clear differentiation promoting activity of pomegranate extracts P and W, representing, respectively, flavonoid-rich or polyphenol fractions from pomegranate fermented juice and pericarp. The activity of the fresh pomegranate juice polyphenols (fraction J) was considerably milder. This shows that very good activity is available from the peels (fraction P), probably owing to the high levels of flavonoid and perhaps also ellagitannin compounds. Comparable activity obtains from the fermented

juice (fraction W), but milder activity from the fresh juice (fraction J). One explanation for this difference between fractions W and J is that in J most of the flavonoids are presumably bound to sugar moieties, but in W have been freed because of the sugar-consuming effect of fermentation.

Flavonoids are examples of secondary metabolites<sup>40</sup> that plants have evolved for their own purposes. Curiously, Cytolenin A, a plant growth regulatory compound,<sup>41,42</sup> promotes differentiation in human cancers, while flavonoids may also help prevent cancers in the plant.<sup>43</sup> Flavonoids, as steroid-mimetic compounds, may act in part through cross-talk with cytokines,<sup>44</sup> to affect differentiation and other processes in human cancer cells. An example of such a cytokine is tumor necrosis factor- $\alpha$ , which elsewhere has been shown to be central to the antioxidative mechanism of fermented pomegranate juice polyphenols.<sup>45</sup>

In summary, these findings confirm a pro-differentiating effect in human HL-60 leukemia cells of pomegranate extracts W and P that is greater than the effect of a compara-



**FIG. 5.** Effect of pomegranate fermented juice polyphenols (fraction W), pomegranate pericarp (peel) polyphenols (fraction P), and pomegranate fresh juice polyphenols (fraction J) on proliferation (PR) of HL-60 human leukemia cells.

ble extract J derived from fresh pomegranate juice. Overall, this capability reinforces the conclusions from our earlier studies that pomegranate fermented juice and pericarp polyphenolic extracts possess heuristic potential as cancer chemopreventive and adjuvant therapeutic agents.

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