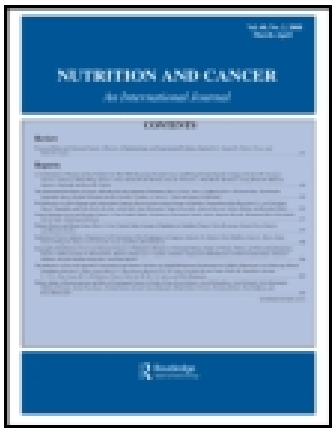

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Bioactive Actions of Pomegranate Fruit Extracts on Leukemia Cell Lines In Vitro Hold Promise for New Therapeutic Agents for Leukemia

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Studies suggest that pomegranates contain bioactive chemicals with potential for treatment and prevention of cancer. Pomegranate juice extracts (PJE) have been shown to inhibit cellular proliferation and tumor growth and induce cell death via apoptosis in a number of cancer cell lines. However, to date, few studies have investigated the potential of PJE in the treatment of leukemia. We investigated the potential effect of PJE on induction of apoptosis and inhibition of cellular proliferation in 8 leukemia cell lines (4 lymphoid and 4 myeloid) and nontumor hematopoietic stem cells (control cells). Apoptosis was assessed by 2 methods: Annexin V-FITC/propidium iodide staining with flow cytometric analysis and 4′-6-diamidino-2-phenylindole (DAPI) morphological assessment. Cell cycle stage was investigated using propidum iodide staining of DNA content and flow cytometric analysis. Live cell counts were also performed using a trypan exclusion assay. PJE significantly induced apoptosis in all cell lines, including nontumor control cells, although lymphoid cells and 2 of the myeloid cell lines were more sensitive. Furthermore, PJE induced cell cycle arrest. These results were confirmed by DAPI analysis and viable cell counts using trypan blue exclusion assay. Our results provide evidence that PJE contain bioactive compounds that could be used in the treatment of leukemia.

INTRODUCTION

Leukemia is defined as a clonal malignant disorder of white cells in the blood and blood-forming organs. Leukemia is characterized by uncontrolled proliferation of white cells, resulting in bone marrow failure and decreased production and function of normal hemopoietic cells. From the time of Virchow's initial discovery, the overall classification of leukemia has become more complex (1). Currently, leukemia is classified based on the predominant cell of origin (myeloid or lymphoid) and the rate of progression (acute or chronic), together with numerous sub-classifications (2).

Leukemia is a major problem worldwide, affecting millions of people each year (3). In the UK, leukemia is the most common childhood cancer, and, overall, leukemia shows a slightly higher incidence in males than females (3). In 2008, it was estimated that 4,367 deaths were caused from leukemia in the UK, and it was ranked as the 11th most common cause of death from cancer (3).

Treatment of leukemia depends on many factors, such as histologic type of leukemia, its stage, and prognostic features (patient's age and overall health) (4). However, current treatment options such as chemotherapy, bone marrow transplantation, and radiotherapy still have several limitations—not least, the cytotoxicity of these therapies to normal cells and the fact that certain chemotherapy agents may cause bone marrow toxicity (5,6) and organ damage (7). In addition, the major causes of treatment failure in leukemia are drug resistance and metastasis to other tissues (8). A number of groups worldwide are investigating new cancer treatment strategies using natural plant products. Evidence is developing on the basic mechanisms of action using in vitro culture and animal models as well as human trials in a number of solid tumors which have shown responses on important markers of disease progression (9,10).

Pomegranate (Punica Granatum) is an ancient fruit that has been used in alternative medicine in many cultures (11). Poylphenolic compounds make up the highest proportion of phytochemicals in pomegranate, which are characterized by multiple phenol rings that bear a number of hydroxyl groups (12). The 2 major types of polyphenolic compounds found in pomegranates are hydrolyzable tannins, which account for the majority of antioxidant activity of the fruit, and anthocyanins (13). The cellular antioxidant activity and total phenolic contents of pomegranate have been shown to be higher than commonly

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consumed fruits such as blueberry, apple, red grape, cherry, and lemon (14).

Pomegranate has shown potential in the treatment of solid tumors, where studies have demonstrated that pomegranate extracts inhibit the proliferation of colon (15), breast (16), lung (17), and prostate (18) cancer cells in in vitro culture. Dietary supplementation with pomegranate extracts inhibited the growth of tumors including skin (19), prostate (20), and lung (17). In terms of induction of apoptosis, pomegranate extracts have been shown to induce apoptosis in prostate and colon cancer cell lines (20,21). Furthermore, in a phase II clinical trial, pomegranate juice has shown promising results against prostate cancer (22). Few studies have investigated the potential of pomegranate juice extract (PJE) in the treatment of leukemia. Kawaii and Lansky (2004) evaluated the effect of fresh and fermented pomegranate juice on HL-60 human leukemia cell line differentiation. Their study demonstrated that treatment with PJEs induced differentiation of HL-60 cells and inhibition of cellular proliferation (23). No studies to date have investigated the effects of PJEs on nontumor cells, and thus selective toxicity to cancer cells has not previously been investigated.

In this study, we investigated the hypothesis that PJE can induce cell death and prevent cellular replication to a greater extent in leukemia cells than nontumor control cells.

MATERIALS AND METHODS

Preparation of PJE

Fresh pomegranates were purchased from Sainsbury's, Ltd. (Sainsbury's, Sheffield, UK). Pomegranates were washed, peeled, and the edible part separated from the pith. Edible parts were juiced using pulp ejector juicer (L'EQUIP), generating 1 ml juice per 1.73 g pomegranate fruit. The pomegranate juice extract was then centrifuged at 1,000 *g* for 35 min and filtered though 0.22 μm sterile syringe filters (Invitrogen, Paisley, UK) to remove any fiber. Before treatment, PJE was diluted (1:1) (v/v) with complete media RPMI (Invitrogen, UK) and recentrifuged at 1,000 *g* for 15 min to remove any precipitate.

Cell Culture

All human leukemia cells were obtained from American Type Culture Collection (ATCC; Middlesex, UK). In this study, we used 4 lymphoid leukemia cell lines: Jurkat (peripheral blood T cell leukemia) (ATCC: TIB-152), SUP-B15 (acute lymphoplastic leukamia) (ATCC: CRL-1929), MOLT-3 (acute lymphoplastic leukaemia, taken from a patient who had received prior multidrug chemotherapy) (ATCC: CRL-1552), and CCRF-CEM (acute lymphoplastic leukemia) (ATCC: CCL-119), and 4 myeloid leukemia cell lines: HL-60 (human promyelocytic leukemia) (ATCC: CCL-240), THP-1 (acute monocytic leukemia) (ATCC: TIB-202), K562 (chronic myloid leukemia) (ATCC: CCL-243), and KG1a (acute myelogenous leukemia)). Normal human hematopoietic stem cells were obtained from Lonza (Slough, UK) and are a primary commercial CD133 +ve population of HSCs obtained from cord blood (LONZA, Slough, UK); these cells were used as a control population of noncarcinogenic primary cells to investigate the effects on hematopoietic stem cells, which are often the cell types affected in leukemias. All cells were cultured in T-75cm^2 flasks (Invitrogen, Paisley, UK) at a cell density of 2×10^6cells /ml in RPMI 1640 medium (Invitrogen, Paisley, UK) supplemented with 10% (v/v) fetal bovine serum, 1.5 mM L-glutamine, and 100 μg/ml penicillin/streptomycin (complete RPMI) and incubated at 37 °C with 5% CO_2.

Following expansion in 75 cm^2 flasks cells were centrifuged at 400 *g* for 10 min, the supernatant was removed, and the pellets resuspended in 5 ml of complete RPMI media. Cells were then seeded in 12-well plates (Fisher Scientific, Leicestershire, UK) at a cell density of 0.5×10^6 cells per well and treated with PJE at concentrations [0% (v/v), 6.25% (v/v), and 12.5% (v/v)] PJE/media in triplicate for 24 and 48 h (all treatments contained equal concentration of FCS of 5% final volume of treatment). Together with additional water controls where physiological media was diluted in sterile distilled water to match each PJE treatment, pH was also adjusted to test effects of the slight acidity induced by PJE using 1% HCl to match pH of PJE concentration. Following treatment, cells were investigated for induction of apoptosis and cell cycle arrest.

Assessment of Apoptosis

The effect of PJE on induction of apoptosis was assessed by 2 methods: Annexin V-FITC/propidium iodide (PI) staining with flow cytometric analysis and 4′-6-diamidino-2-phenylindole (DAPI) morphological assessment.

Annexin V FITC /PI Stain Flow Cytometric Assay

After treatment of cells with PJE at concentrations of 0%, 6.25%, and 12.5% (v/v) for 24 and 48 h, cells were harvested into 1.5 ml Eppendorf tubes and centrifuged at 400 *g*, 4°C for 5 min. Supernatant was removed and cells washed once in 100 μl cold Dulbecco's phosphate buffered saline (DPBS) (Invitrogen, Paisley, UK). Following washing, supernatant was removed and cells resuspended in 100 μl binding buffer (BB) (10 mM HEPES/NaOH, pH7.4, 140 mM NaCl, 2.5 mM CaCl2) (Beckton-Dickinson, Oxford, UK). After washing with BB twice, cells were resuspended in 50 μl of BB and 5 μl of Annexin V FITC (Beckton-Dickinson, Oxford, UK). Cells were mixed gently and incubated for 15 min in the dark. Labeled cells were transferred to FACS tubes, and 300 μl of PI (50μg/ml) (Sigma, Poole, UK) was added and mixed gently; samples were then read directly on flow cytometer [FACS Calibur cytometer (Becton-Dickinson, UK)]. Data were recorded from 10,000 cells per sample and analyzed using FlowJo software (Tree Star, Ashland, OR).

Annexin V FITC is used in conjunction with PI to allow identification of early apoptotic, late apoptotic, and necrotic cells. Live cells are Annexin V/FITC and PI negative; cells that are in early apoptosis are Annexin V-FITC positive and PI

negative and cells that died through apoptosis or necrosis are both Annexin V-FITC and PI positive and are referred to as dead within this study; the distinction between late apoptosis and necrosis is not possible with this technique.

DAPI Stain

Following treatment with PJE for 24 and 48 h, cells were transferred to Eppendorf tubes, collected by centrifugation for 5 min at 400 *g*, 4°C, and washed in 100 μl of cold DPBS twice. Cells were then resuspended in 100 μl 4% (v/v) paraformaldehyde and stored overnight at 4°C. Following fixation, cells were transferred to slides via a 20-min cytospin at 1,000 rpm (Shandon Cytospin 3 Centrifuge). Slides were then dried at room temperature and 100μl of DAPI stain (Sigma, Poole, UK) applied to cells for 10 min in the dark at room temperature. Excess DAPI stain was removed and slides mounted in 90% glycerol and coverslips applied and sealed with nail varnish. An Olympus BX60 fluorescence microscope was used to assess the morphology of cell nuclei at excitation wavelength 350 nm. Living cells are characterized by round, clear edges. Apoptotic cells were identified by condensed chromatin, irregular edges around the nucleus, and increased number of fragmented nuclear bodies. This technique allows calculation of apoptotic cells excluding cells that die via necrosis as these cells show clear morphological differences.

Effect of PJE on Cell Proliferation

The effect of PJE on cell cycle was investigated using a PI stain, and analyzed using flow cytometry. In addition, trypan blue exclusion assay to determine the number of viable cells following PJE treatments was performed.

Cell Cycle Analysis

After 24 h incubation with PJE, cells were centrifuged for 5 min at 400 *g*, 4°C, washed twice in 100 μl ice-cold DPBS. Following washing, cells were stored at −20°C in 80% ethanol overnight. Fixed cells were then washed twice with 100 μl cold DPBS and resupended in 50 μl of RNase A (0.1 unit/ml) (Sigma, Poole, UK) and 300 μl of PI (50 μg/ml) (Sigma, Poole, UK). Cells were incubated at 4°C overnight prior to analysis with FACS Calibur cytometer. Data from 10,000 cells per sample were recorded and processed using FlowJo software and Waston (pragmatic) analysis of cell cycle (Tree Star, Treestar, Ashland, OR).

Trypan Blue Exclusion Assay

Trypan blue exclusion assay was used to quantify the number of live cells in suspension culture following treatment with PJE at a concentration 0% (v/v), 6.25% (v/v), and 12.5% (v/v) for 24 and 48 h to determine effects of PJE on total live cell number.

Statistical Analysis

Data are presented as the mean ± SEM and were analyzed by Stats Direct using Shapiro Wilke test to check for normality. Data were shown to be nonparametric, and thus a Kruskal-Wallis and Connover-Inman post hoc test was used to test for significance. $P \leq 0.05$ was considered significant.

RESULTS

Effect PJE on Induction of Apoptosis Using Annexin-FITC/PI

A significant decrease in the number of live cells and a significant increase in the number of apoptotic and dead cells in a dose-responsive manner was observed in all cells following 24 and 48 h. However, the sensitivity to PJE between leukemia cell types and nontumor primary HSCs was observed. CCRF-CEM (derived from a patient with acute lymphoblastic leukemia) was the most affected cell line, where 2.1% of cells remained viable following 6.25% (v/v) PJE and 0.02% viable following 12.5% (v/v) PJE treatment for 24 h (Fig. 1). In general, lymphoid leukemia cell lines were more affected than myeloid cell lines.

KG-1a (derived from a patient with acute myelogenous leukaemia) was the most sensitive to PJE of the myeloid cell lines investigated, with percentage of viable cells decreasing to 31% following 6.25% PJE and 6.25% following 12.5% PJE treatment after 24 h ($P \leq 0.05$). THP-1, although the least affected leukemia cell line, also demonstrated significant decrease in percentage of viable cells but was less sensitive with 63% remaining viable following 12.5% PJE treatment for 24 h ($P \leq 0.05$). The nontumor HSC primary cells displayed similar sensitivity patterns to the least sensitive leukemia cell line THP-1 cells (Fig. 1).

Nontumor HSCs were significantly less sensitive ($P \leq 0.05$) than the majority of leukemia cell lines at all doses and durations of treatment, with the exception of THP-1 and K562 leukemia cell lines ($P > 0.05$) (Fig. 2). Nontumor HSCs were significantly more sensitive than THP-1 cells following 6.25% (v/v) PJE after 48 h: however, at the higher dose of 12.5%, PJE THP-1 were affected significantly more than nontumor HSCs ($P \leq 0.05$). K562 showed similar sensitivity to nontumor cells at low dose following 24 h and 48 h but was more sensitive at higher dose of PJE ($P \leq 0.05$) (Fig. 2).

Effect of Isotonic/Osmolarity and pH Changes on Induction of Apoptosis

To ensure the toxic effect observed from PJE was not purely a result of altered isotonic/osmolarity or decreased pH induced by the inclusion of PJE, cells were treated with an equivalent concentration of distilled water in place of PJE or in pH adjusted media [pH of control media = 7.5; PJE at 6.25% (v/v) = 7.42; pH of PJE at 12.5% (v/v) = 7.23]. The majority of cell types investigated showed no significance decrease in live cells or increase in the apoptotic or dead population (late apoptotic and necrotic) following inclusion of water controls (Fig. 3A) or in altered pH (Fig. 3B) ($P \leq 0.05$). A small but significant decrease in live cells was observed in THP-1 cells treated with water or pH equivalent to 12.5% PJE, and in CCRF-CEM treated with

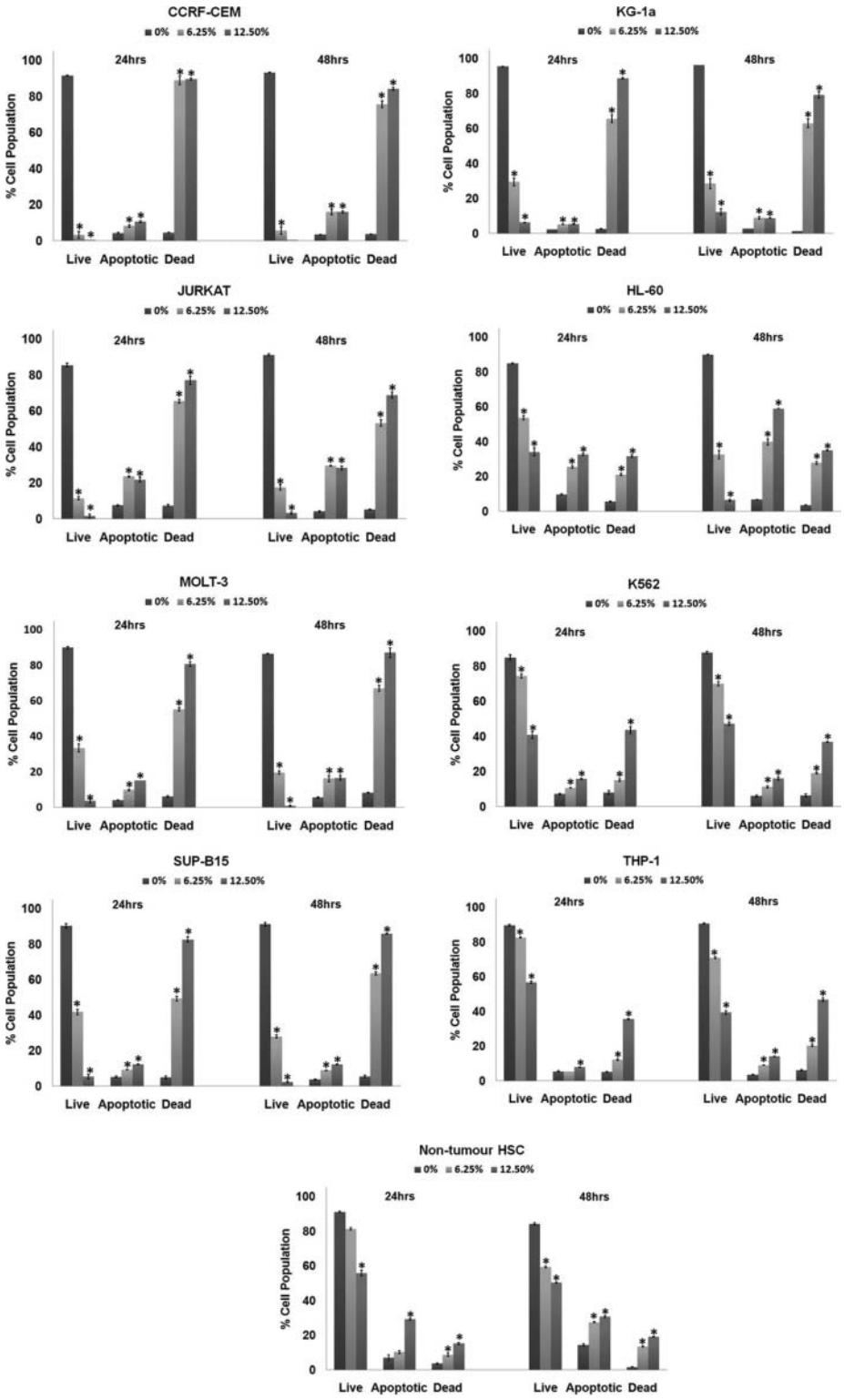

FIG. 1. Analysis of Annexin V-FITC/propidium iodide (PI) following treatment with pomegranate juice extracts (PJE) (0%, 6.25% and 12.5%) for 24 and 48 h on CCRF-CEM (acute lymphoplastic leukemia), Jurkat (peripheral blood T cell leukemia), MOLT-3 (acute lymphoplastic leukemia patient released following chemotherapy), SUP-B15 (acute lymphoplastic leukemia), KG1a (acute mylogenous leukemia), HL-60 (human promyelocytic leukemia), K562 (chronic myloid leukemia) THP-1(acute monocytic leukemia) and CD133 positive hematopoietic stem cells (nontumor HSC). (Mean ± SEM, $^{*}P \leq 0.05$ vs. untreated control).

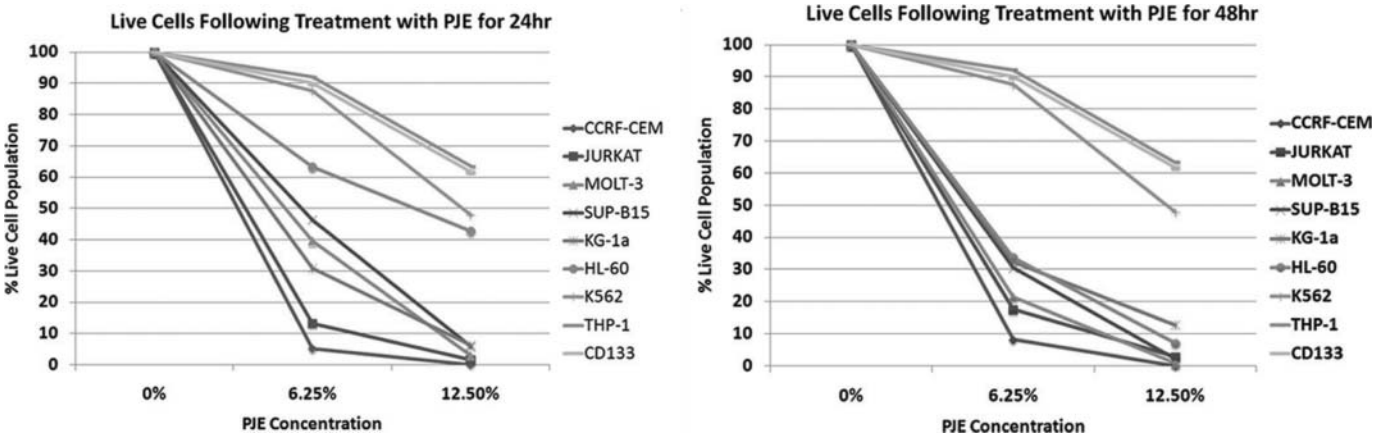

FIG. 2. Live cell populations normalized to untreated controls in 4 lymphoid leukemia cell lines (CCRF-CEM, JURKAT, MOLT-3, SUP-B15), 4 myeloid leukemia cell lines (KG-1a, HL-60, K562, THP-1), and non-tumor hematopoietic stem cells (CD133) following treatment with pomegranate juice extracts (PJE) for 24 and 48 hrs.

pH adjusted media (Fig. 3); however, it is noteworthy that these effects were far smaller than those seen following PJE treatments (Fig. 1).

Effect PJE on Induction of Apoptosis Using DAPI Staining

Nuclear morphology of treated cells confirmed Annexin V-FITC/PI data with a decrease in the percentage of cells displaying normal nuclear morphology in all cell types and an increase in the number of cells displaying apoptotic morphology (Fig. 4). Sensitivity patterns matched those seen using Annexin V-FITC/PI analysis with (CCRF-CEM > Jurkat > MOLT 3 = SUP-B15 = KG-1a > HL-60 > K562 = THP-1 = nontumor HSCs) (Fig 2).

Effect of PJE on Cell Cycle

Differential effects on cell cycle stage following PJE treatment were observed dependent on cell type investigated and dose and duration of PJE treatments. Following 6.25% PJE treatment for 24 h, all cells displayed arrest of cell cycle in G_0/G_1 phase although this effect was only significant in Jurkat, MOLT-3, HL-60, and THP-1 cells ($P \leq 0.05$). At a concentration of 12.5% (v/v), PJE the majority of leukemia cell lines displayed significant S-phase arrest ($P \leq 0.05$), with the exception of HL-60 and KG-1a cells, where a small G_0/G_1 arrest was observed ($P \leq 0.05$) (Fig 5). Nontumor HSCs also induced G_0/G_1 arrest at 6.25% PJE treatment and S-phase arrest in cells treated with a higher dose of 12.5% ($P \leq 0.05$) (Fig. 5).

Effect of PJE on Viable Cell Number

Treatment with PJE at concentrations of 6.25% and 12.5% resulted in a significant decrease in the number of viable cells compared to untreated cells (0%) following 24 and 48 h incubation ($P \leq 0.05$). Nontumor HSC cells also showed a significant decrease in the number of viable cells at 6.25% and 12.5% PJE following 24 and 48 h incubation (Fig. 6). However, sensitivity as for apoptosis analysis showed lower sensitivity than the lymphoid leukemia cell lines (CCRF-CEM, JURKAT, MOLT-3, and SUP-B15), and 2 of the myeloid cell lines (KG-1a and HL-60). K562 and THP-1 cells displayed similar sensitivity compared to nontumor cells.

DISCUSSION

Here, we investigated whether PJE contains bioactive agents that may be beneficial in the treatment of leukemia. The effect of pomegranate extracts and its purified constituents have been investigated previously using cell lines derived from solid tumors; few, however, have investigated the effect in leukemia cells. Khan et al. (2009) investigated the effect of PJE on induction of apoptosis in 2 breast cancer cell lines (SUM 149 and MDA-231), together with nontumor MCF-7 cells, where treatment with PJE induced apoptosis following 72 h selectively within the 2 tumor cell lines and did not affect the nontumor MCF-7 cells (24). Here we also identified an increased sensitivity to PJE within the majority of tumor cell lines compared to nontumor hematopoietic stem cells. Together, these studies suggest that pomegranate extracts could hold promise for selective cancer therapies in subsets of cancer types.

Kasimsetty et al. (2010) demonstrated induction of apoptosis in colon cancer cell line (HT-29) induced by pomegranate juice derived ellagitannins and urolithins following 24 h (25). Malik et al. (2005) also demonstrated induction of apoptosis, this time in prostate cancer cells (PC3) (20). Here, we have shown that PJE can also induce apoptosis in a number of leukemia cell lines. Importantly, we have shown that the response to PJE varied between leukemia cell lines and the majority were affected to a greater extent than nontumor control cells. The sensitivity to chemotherapy agents often displays selective targeting to either leukemias of the lymphoid or myeloid origin; for example, phenoxodiol shows greater toxicity to lymphoid leukemias than myeloid leukemias (26), as do extracts from carrot juice (27).

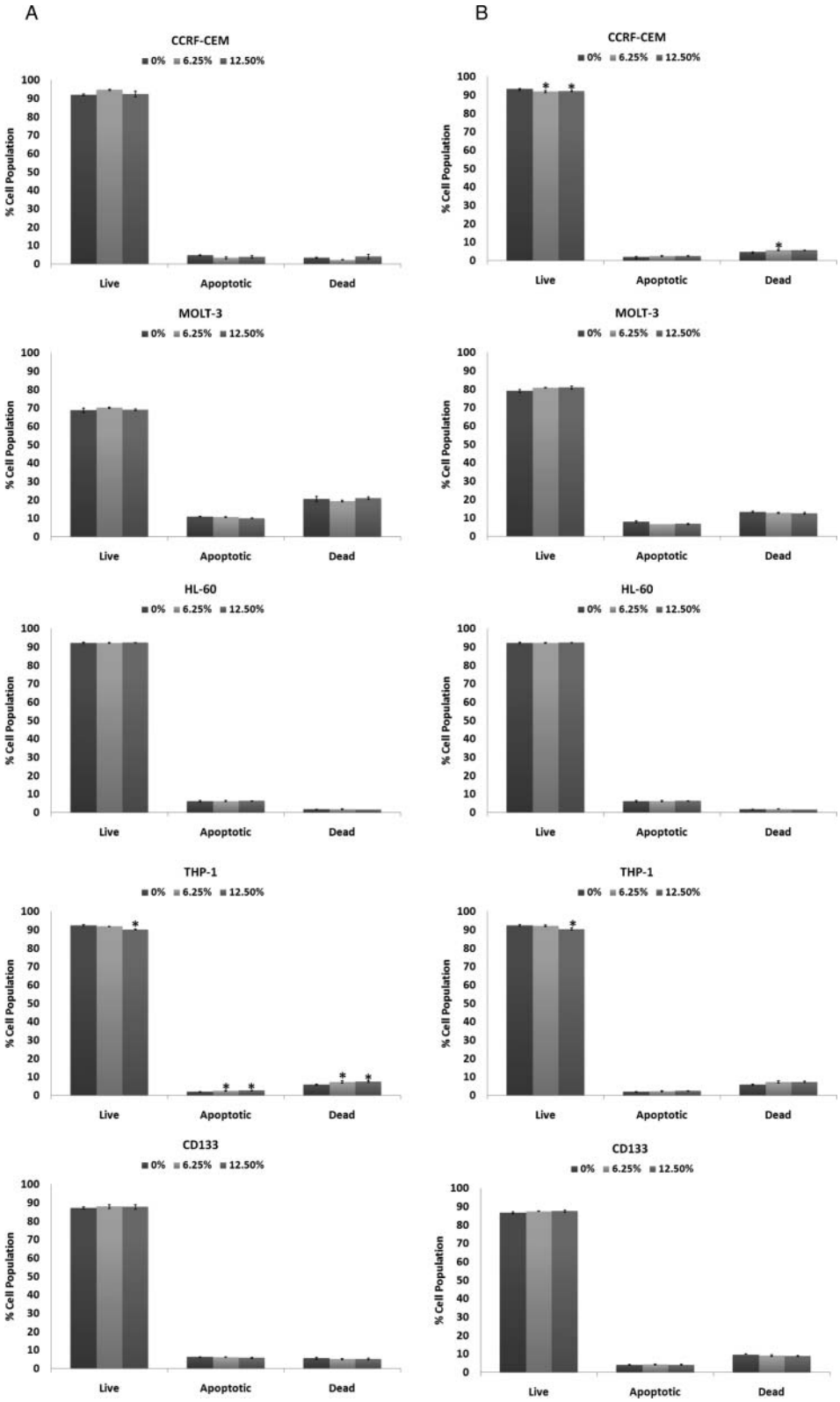

FIG. 3. Annexin V-FITC/PI staining detected by using flow cytometry. A: CCRF-CEM (acute lymphoplastic leukemia), MOLT-3 (acute lymphoplastic leukemia patient released following chemotherapy), HL-60 (human promyelocytic leukemia), THP-1 (acute monocytic leukemia) and CD133 positive hematopoietic stem cells treated with equivalent concentration of water. (Mean ± SEM, $^{*}P \leq 0.05$ vs. untreated control). B: CCRF-CEM (acute lymphoplastic leukemia), MOLT-3 (acute lymphoplastic leukemia patient released following chemotherapy), HL-60 (human promyelocytic leukemia), THP-1 (acute monocytic leukemia), and CD133 positive hematopoietic stem cells treated in Ph adjusted media. (Mean ± SEM, $^{*}P \leq 0.05$ vs. untreated control).

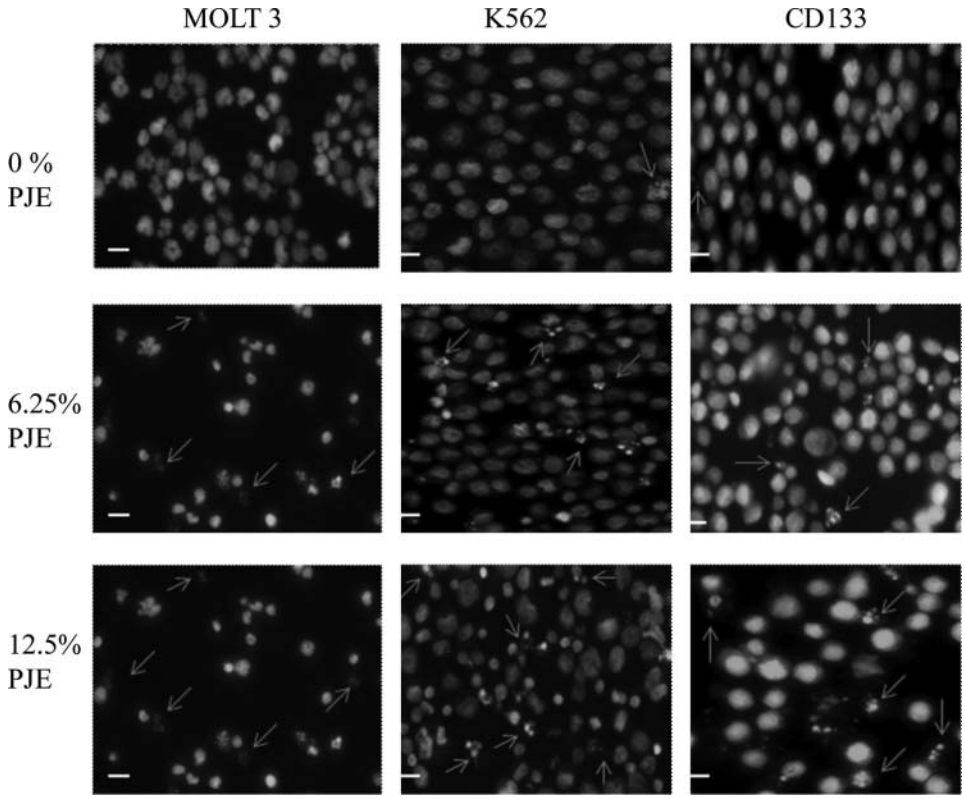

FIG. 4. Analysis of apoptosis using DAPI staining following treatment with pomegranate juice extract (PJE) at 0%, 6.25%, and 12.5% for 24 h on CD133 (positive hematopoietic stem cells), MOLT-3 (acute lymphoplastic leukemia patient released following chemotherapy), and K562 (chronic myeloid leukemia). (Example of apoptotic cells indicated by →) (scale bar = 20 μm).

Lymphoid leukemias and myeloid leukemias are well known to be caused by abnormalities to differential signaling pathways; for example, STATs are involved in the pathogenesis of myeloid leukemias and act as a target for selective chemotherapy agents such as the tyrosine kinase inhibitors, which do not show any effect within lymphoid leukemias (28). Thus it is not surprising to see differential sensitivity patterns between cell lines derived from myeloid and lymphoid leukaemias within the current study. Future work will focus on investigating the molecular mechanisms of PJE on these cell lines to elucidate further the molecular targets of PJE and hence identify reasons for the selective targeting toward lymphoid leukemia cell lines. Interestingly, however, some of the myeloid leukemia cell lines did show cytotoxic effects, suggesting that in certain subtypes of myeloid leukemia PJE may still be a useful therapeutic agent.

In addition to induction of apoptosis, cell cycle arrest can be a useful target for cancer therapies (29). Here we demonstrated a dose-dependent arrest of cell cycle at different phases. At low doses of PJE, the majority of cells showed G_0/G_1 arrest, suggesting induction of senescence (29). G_0/G_1 arrest has been observed previously in a number of tumor cell lines following PJE treatments; for example, human lung carcinoma A549 cell line treatment with PJE for 72 h resulted in a dose-dependent arrest of cells in G_0/G_1 phase of the cell cycle (17). In addition, Kasimsetty et al. observed a reduction in S phase and an accumulation of cells in G_0/G_1 in DU145 colon cancer cells following PJE treatment (25). This reduction in S phase and accumulation of cells in G_0/G_1 was also observed within the higher concentration of PJE for HL-60 and KG-1a cell lines, which were also the most sensitive myeloid cell lines to induction of apoptosis within our study. Interestingly, these 2 cell lines were the only ones investigated that at this concentration induced G_0/G_1 arrest; these 2 cell lines show myeloblast morphology unlike the other 2 myeloid cell lines that appears to affect the cell cycle response. This may be due to modulation of c-myc expression that is overexpressed within HL-60 cells and inhibition of c-myc induces G_0/G_1 cell cycle arrest (30); thus it is possible the G_0/G_1 arrest observed here is due to inhibition of c-myc expression.

In all other cell lines and nontumor hematopoietic stem cells treated with 12.5%, PJE S-phase arrest was observed, demonstrating again variability between cell lines from different leukemia types. This effect has been observed previously in Caco-2 cells treated with the pomegranate extracted ellagitannin: punicalagin (26). Kasimsetty et al. (25) also evaluated the effect of PJE-derived ellagitannins (gallic acid, ellagic acid, gallagic acid, hexahydroxydiphenic, gallagyldilactone, punicalins, and punicalagins) on HT-29 colon cells, and once again S-phase

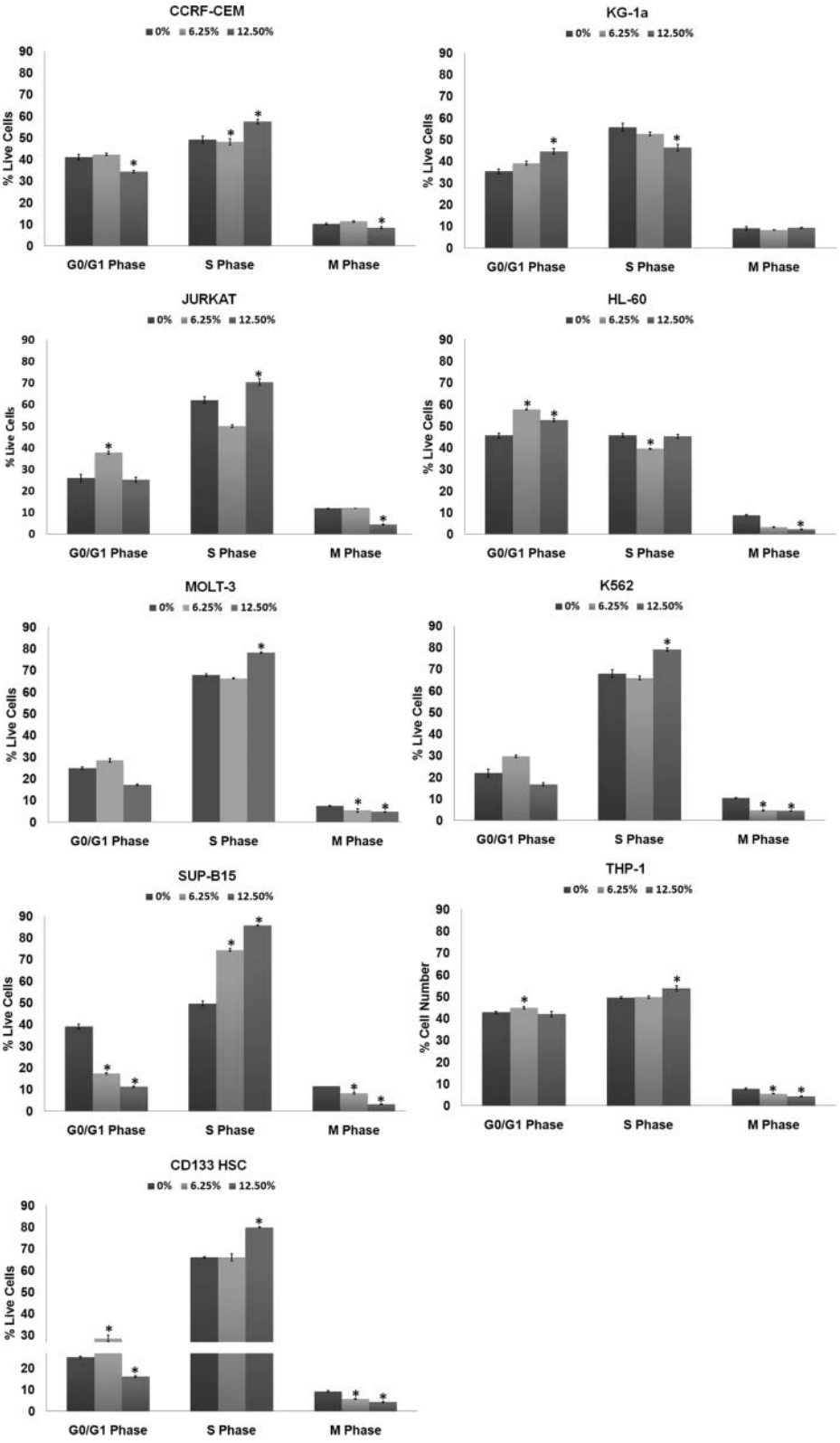

FIG. 5. Analysis of cell cycle using flow cytometry following treatment with pomegranate juice extract (PJE) at concentration 0%, 6.25%, and 12.5% for 24 h incubation on CCRF-CEM (acute lymphoplastic leukemia), Jurkat (peripheral blood T cell leukemia), MOLT-3 (acute lymphoplastic leukemia patient released following chemotherapy), SUP-B15 (acute lymphoplastic leukemia), KG1a (acute mylogenous leukemia), HL-60 (human promyelocytic leukemia), K562 (chronic myloid leukemia), THP-1 (acute monocytic leukemia), and CD133 positive hematopoietic stem cells. (Mean ± SEM, $^*P \leq 0.05$ vs. untreated control).

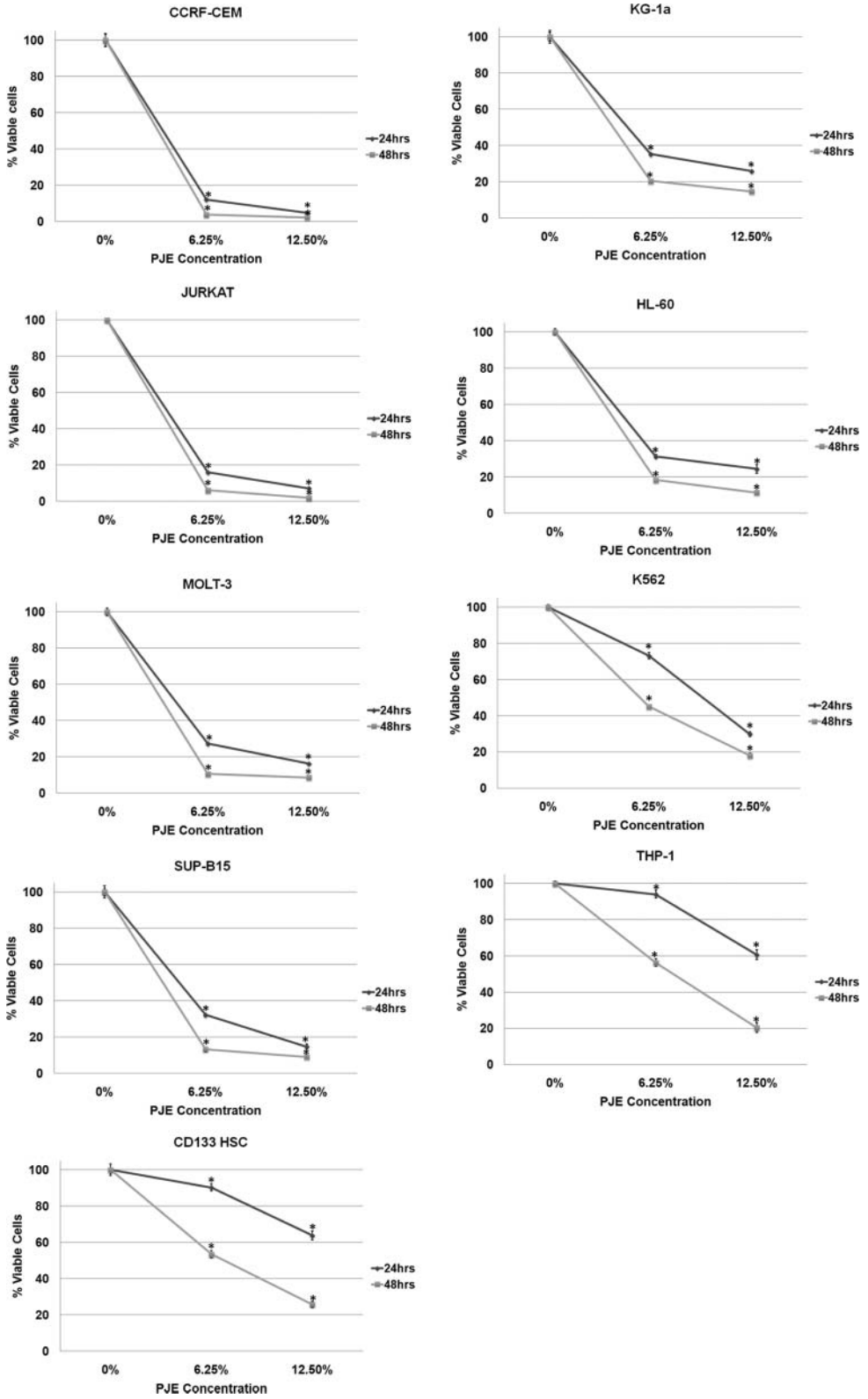

FIG. 6. Effect of pomegranate juice extract (PJE) on cell viability using trypan exclusion assay following 24 and 48 h incubation at 6.25%, 12.5%, and 25% on CCRF-CEM (acute lymphoplastic leukemia), Jurkat (peripheral blood T cell leukemia), MOLT-3 (acute lymphoplastic leukemia patient released following chemotherapy), SUP-B15 (acute lymphoplastic leukemia), KG1a (acute mylogenous leukemia), HL-60 (human promyelocytic leukemia), K562 (chronic myloid leukemia), THP-1 (acute monocytic leukemia), and CD133 positive hematopoietic stem cells. (Mean ± SEM, $^*P \leq 0.05$ vs. untreated control). Average cell number of untreated cells was set at 100% and relative number of cells calculated accordingly. (Mean ± SEM, $^*P \leq 0.05$ vs. untreated control).

arrest was observed. Differential effects on cell cycle dependent on concentration or duration has been seen in a number of studies previously—for example, Mertens-Talcott et al. (31) investigated 3 polyphenolic compounds (camptothecin, ellagic acid, and quercetin) on a lymphoid leukemia cell line (MOLT-4); they demonstrated G_0/G_1 arrest following camptothecin treatment for 12 h, but this effect was not observed after later time points, whereas treatment with a combination of ellagic acid and quercetin showed no effect following 12 h, but S-phase arrest was observed following 24 h (31). S-phase arrest can result from 2 main causes, either an artifact of cells undergoing apoptosis within G_2/M phase thus reducing their DNA content and accumulating within the S-phase peak during cell cycle analysis. Alternatively, agents that alter cell cycle expression that control progression through S phase will result in S-phase arrest. This has been shown previously in colon cancer cells treated with 2,3-dichlorophenoxypropyl, where S-phase arrest was induced by overexpression of p21 (32) or by affecting agents involved in DNA repair mechanisms such as inhibition of Poly(ADP-ribose) as seen following treatment of RAW 264.7 macrophage cell line with gallotannin (33), which could be a mechanism induced by pomegranate extracts within our current study.

Within this study, we also investigated the overall effect of PJE on total viable cell number, which shows a combined effect of induction of apoptosis and inhibition of cellular proliferation, confirming our previous data. Decreased number of viable cells has previously been demonstrated following pomegranate extract treatments in colon, prostate, and breast cancer cell lines (15,20,24,34–37).

PJE investigated within this study was a total extract of all the polyphenolics and anthrocyanins together with any other potential bioactive agents, rather than a pure fraction—this was performed to investigate the effects of combined responses, rather than simple individual actions. Pomegranates contain a wide number of potential bioactive agents, including hydrolyzable tannins (such as punicalin, punicalagin, and gallagic), and anthocyanins (such as delphmidin, cyaniding, and pelargonidin), which can often act synergistically or indeed inhibit the actions of other agents (26,31). Future work will investigate fractionation of the pomegranate juice extracts and purification to identify the bioactive compounds responsible for the actions seen within our study.

Our study is the first to investigate the effect of PJE on 8 different leukemia cell lines in addition to normal hemopoietic stem cells; we demonstrated important variations in sensitivity between different types of leukemia cell lines and normal cells. Our study suggests that the active components from PJE could have potential as agents in the treatment of certain leukemias, and further work will aim to identify these components.

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