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Anticancer Activities of Pomegranate Extracts and Genistein in Human Breast Cancer Cells

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ABSTRACT Previous studies have demonstrated the anticarcinogenic activity of pomegranate extracts and genistein in a series of human cancer cells. In the present study, the potential anticancer effects of pomegranate extracts and genistein on inhibition of cell proliferation and induction of apoptosis in human breast cancer cells was investigated. Human breast cancer cells (MCF-7) were cultured as monolayers in complete RPMI 1640 medium. The cells were cultured for 48 hours to allow growth and achieve about 80% confluence in 48-well culture plates, and then exposed to the agents for 24 hours in single and combination treatments. Post-treatment growth rate and apoptosis induction were assessed by the use of a series of bioassays—lactate dehydrogenase and 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium (inner salt) for viability and cytotoxicity; acridine orange-ethidium bromide and terminal deoxyribonucleotidyl transferase-mediated dUTP nick-end labeling assays for induction of apoptosis. Both pomegranate extracts and genistein had significant (dose- and time-dependent) cytotoxic and growth inhibition effects on MCF-7 cancer cells. Both growth inhibition and cytotoxicity were significantly higher ($P < .01$) in the combination treatments than in the single treatments with either agent. The data revealed that both drugs in single and in combination treatments induced apoptosis in MCF-7 cells. Apoptotic induction in the combination treatments was significantly higher ($P < .01$) than in single treatments. Both pomegranate extracts and genistein inhibit the growth of MCF-7 breast cancer cells through induction of apoptosis, with combination treatment being more efficacious than single treatments.

KEY WORDS: • ***apoptosis*** • ***chemosensitivity*** • ***genistein*** • ***human MCF-7 breast cancer cells*** • ***pomegranate***

INTRODUCTION

BREAST CANCER IS THE MOST COMMON FORM of cancer (other than skin) and the leading cause of cancer mortality among women, next to lung cancer, in the United States.[1] Each year, 182,000 women are diagnosed with breast cancer, and 43,300 die[2]; also, 1,600 men are diagnosed with breast cancer, with 400 fatalities.[2] Breast cancer, like many other cancers, tends to spread throughout the body without any symptom. By the time a tumor is detected, there is a high probability that metastatic lesions will be present.[3–6] This observation has generated significant interest in the search for novel anticancer agents.[3,4,6,7] Attention is currently focused on therapeutic regimens that are primarily based on apoptosis induction in the cancer cells, in an attempt to significantly decrease obnoxious side effects. Natural products/plants have been the main focus.

Although natural products have long been a fertile source of cures for cancer, there has been a desperate and continuous need for development of new anticancer drugs and chemotherapy strategies aimed at killing both primary and metastatic cancer cells. Pomegranate extracts and the isoflavone genistein (4′,5′,7-trihydroxyisoflavone) have been receiving attention as anticancer agents.[7,8] The pharmacological properties of pomegranate extracts have been analyzed. The oil polyphenols inhibit eicosanoid enzymes, including cyclooxygenase and lipoxygenase.[9] Extracts from the flowers lower blood sugar in rodents,[10] and the juice extract inhibits low-density lipoprotein oxidation and atheromatous plaque formation in rodents and humans.[11] The juice contains flavonoids, including anthocyanins and phenolic acids,[12] and the pericarp contains tannins and elagitannins.[13] Because commercial juice is typically obtained by pressing whole fruits, considerable enrichment of the juice by the pericarp polyphenols generally occurs.[8] Flavonoids[14,15] and tannins[16–18] inhibit cancer cell growth *in vitro* and *in vivo*. Furthermore, the pomegranate seed oil contains a very high amount of steroids, including esterone,[19] 17β-estradiol, campestrol,[8] estriol, testosterone, stigmasterol, and sitosterol.[20,21] Many of these compounds have cancer chemopreventive properties, in addition to antimicrobial,[22] antiparasitic,[23] antiviral,[24] and anticancer[25] properties.

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Genistein is a natural isoflavone phytochemical present in soybean. In the gastrointestinal tract, the β-glucoside conjugates of soy are converted by the natural gut microflora into free genistein and other related isoflavones, which are present in circulating blood, accumulate in tissue and are excreted in urine of people who consume high amounts of soy in their diet.[26] Epidemiological studies have revealed that individuals who consume a traditional diet high in soy products have a low incidence of certain types of cancer, such as breast, prostatic, and colon cancer,[27] and other neovascular diseases.[28] Different mechanisms may be involved in the observed chemopreventive and antiproliferative properties of genistein. Some early studies, which focused on the anti-estrogenic activity of genistein, showed its ability to bind *in vitro* sheep uterine estrogen receptors (ERs) and human breast cancer ERs[29,30] and suggested that its chemopreventive activity was the result of interference at the ER level in the tumor-promoting effect of estrogens. Others analyzed the ability of genistein to inhibit protein tyrosine kinases, particularly epidermal growth factor receptor autophosphorylation, by competing with ATP rather than with the protein substrate[31] and indicated that genistein's antiproliferative effect is a result of interference in the tyrosine kinase cascade activated by mitogens. A direct effect of genistein on angiogenesis through inhibition of endothelial cell proliferation[32] may also account for the delay in tumor appearance and especially for the antimetastatic activity.[33] The antiproliferative effect of genistein has also been ascribed to direct inhibition of topoisomerase-II by a nonintercalating irreversible binding to DNA[34] and to induction of apoptosis.

This study was undertaken (1) to determine the chemotherapeutic capabilities of the isoflavone genistein and of pomegranate (*Punica granatum*) extracts in human breast cancer cells (MCF-7) and (2) to investigate a potential therapeutic synergy between the two agents.

MATERIALS AND METHODS

Reagents

Genistein was purchased from Indoline Chemical Co. (Somerville, NJ) and constituted in dimethyl sulfoxide (DMSO) solvent as 10, 20, 30, 50, and 70 mg/mL solutions ($G_{10–70}$) (containing 0.1% DMSO) and frozen at −37°C until used. Pomegranate (*P. granutum*) extracts were donated by Dr. Ephraim Lansky (Rimonest Ltd., Haifa, Israel) and constituted in DMSO as solvent at concentrations of 12, 28, 40, 60, and 80 μg/mL ($P_{12–80}$) (containing 0.1% DMSO) and stored at −37°C. Culture media, RPMI 1640, fetal calf serum, antibiotics, trypsin-EDTA, and other reagents were purchased from Sigma Chemical Co. (St. Louis, MO). Human breast cancer cells (MCF-7) were obtained from the collection of one of the authors (J.K.-D.). Cells were grown and maintained as monolayers in 25-mm^2 tissue culture flasks (Sigma) in RPMI 1640 growth medium, containing 15 m*M* HEPES, and supplemented with 0.45% (wt/wt) glucose, 5.0% fetal calf serum, and 100 U/mL penicillin plus 100 μg/mL streptomycin.

Cell culture

To assess the chemosensitivity of human breast cancer cells to single and combination treatments with genistein and pomegranate extracts, cells were subcultured under 5% CO_2 at 37°C for 48 hours to reach 80% confluence. The cells were harvested by gentle scraping with a cell scraper and resuspended in the medium. From the suspension, 2.5×10^4 cells in 200 μL was dispensed into each well of 24-well microtiter plates (MTPs) and cultured under the same conditions described above for 48 hours to allow adherence of the cells. The supernatants were gently aspirated, and genistein and/or pomegranate extracts were added over a range of five cytotoxic concentrations ($P_{12–80}/G_{10–70}$) in single and combination treatments. In preliminary studies the 50% inhibitory concentration (IC_{50}) of pomegranate extracts averaged 40 μg, and that for genistein was 20 μg. Therefore in the combination treatments, the IC_{50} of one agent was used against varying concentrations of the other. All treated MTPs were incubated at 37°C in a humidified atmosphere of 5% CO_2 in air for a maximum of 72 hours. At 24 and 72 hours of incubation, 100 μL of the supernatant from each well was gently aspirated into microcentrifuge tubes and stored at −37°C until assayed for lactate dehydrogenase (LDH) enzyme activity. At each time point, the media were replenished with equal volumes of the appropriate reagent concentration. At 72 hours of incubation, the cells in each MTP well were processed for the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium, inner salt (MTS) assay and the frozen supernatants for LDH activity.

MTS assay

MTS assay depends on bioreduction of tetrazolium by the mitochondria in live cells into insoluble formazan product in the cell culture medium. MTS is a non-radioactive calorimetric assay that quantifies proliferating cells in a population of cells. It is composed of solutions of a tetrazolium compound (MTS) and an electron-coupling reagent (phenazine ethosulfate). Briefly, following the cell culture as described above, 20 μL of MTS (2.5 mg/mL; Sigma) stock solution was added to each well and incubated for 3 hours under standard conditions of 5% CO_2 and 37°C. The purple formazan product (indicative of reduction of MTS) became visible, and the absorbance was read on a Multiskan® (Labsystems, Helsinki, Finland) Biochromatic automated microplate reader at 490 nm.

LDH assay

LDH activity was measured by a non-radioactive protocol using the LDH cytotox kit (catalogue number 1644 793, Boehringer-Mannheim Biochemica GmbH, Mannheim, Ger-

many). The LDH assay is based on the release of the cytosolic enzyme, LDH, from cells with damaged cellular membranes. Thus, in cell culture, the course of drug-induced cytotoxicity can be followed quantitatively by measuring the activity of LDH in the supernatant. The previously frozen supernatant was thawed for LDH determination. Briefly, 100 μL per well of each cell-free supernatant was transferred in triplicates into wells in a 96-well MTP, and 100 μL of LDH-assay reaction mixture (dye-catalyst mixture from the kit) was added to each well. After a 3-hour incubation under standard conditions, the absorbance/optical density of the color generated was read on a Multiskan biochromatic automatic microplate reader at 490 nm.

Detection of treatment-induced apoptosis

To assess the degree of treatment-induced apoptosis and/or necrosis in MCF-7 cells, the cells were exposed to genistein and pomegranate extracts and cultured as described above. Apoptosis was determined by acridine orange/ethidium bromide nuclear stain, and confirmed by the terminal deoxyribonucleotidyl transferase (TdT)-mediated dUTP nick-end labeling (TUNEL) assay. TUNEL detects apoptosis-specific DNA fragmentation. The treated cells were first washed and resuspended in phosphate-buffered saline for detection of apoptosis as detailed below.

Acridine orange/ethidium bromide. Briefly, 10 μL of the reaction mixture (1:1 acridine orange-ethidium bromide) was added to 250 μL of cell suspension. This was kept in the dark for 20 minutes, after which about 5 μL was dispensed onto microscope slides and examined under a fluorescence microscope. Detection of apoptosis was based on morphological and fluorescent characteristics of the stained cells as previously described.[35] Viable cells were indicated by bright green, apoptotic cells by orange/brown, and necrotic cells by red. Quantitative assessments were made by determining the percentages of apoptotic cells by counting 250 cells in five to seven fields of view.

DNA fragmentation (TUNEL) assay. Apoptosis was further determined/confirmed by TUNEL, using the ApopTag® kit (Boehringer Mannheim Co., Indianapolis, IN) as previously described.[36] The kit reagents detect apoptotic cells *in situ* by specific end labeling and detection of DNA fragments produced by the apoptotic process. Briefly, slides were prepared with aliquots from the cell suspensions (prepared above). The cells (slides) were permeabilized with Triton X-100 at 4°C for 2 minutes and then flooded with TdT enzyme and digoxigenin-dUTP reaction buffer (TUNEL) reagent for 60 minutes at 37°C, followed by further incubation in anti-fluorescein antibody Fab fragments conjugated with alkaline phosphatase. The cells were then stained with chromogenic substrate (Fast Red) and mounted for microscopic analysis under a phase-contrast microscope (BH-2, Olympus, Melville, NY). Negative controls were performed by substituting distilled water for TdT enzyme in the preparation of working solutions. The stained, mounted cells were examined at ×100, ×200, and ×400 magnification of the microscope (Olympus BH-2). Cell death was quantitated by counting 200 cells in five to seven separate fields of view per slide and noting the percentage of apoptotic cells based on morphological appearance, as previously described.[36,37]

Statistical analysis

All the experiments were performed in replicates of three and repeated twice, to confirm similar results. Significance of the differences in the means was determined using Student's *t* test and considering $P < .05$ to be statistically significant.

RESULTS

Pomegranate extracts and genistein inhibit growth and proliferation of human MCF-7 breast cancer cells

The effects of pomegranate extracts and genistein on inhibition of growth and proliferation of human MCF-7 breast cancer cells were determined by using LDH and MTS bioassays. In single and combination treatments, both pomegranate extracts and genistein inhibited cell growth and decreased cell survival through induction of cell death in both a time- and dose-dependent manner. The data obtained revealed that MCF-7 cells were more sensitive to the combination treatment than to the single treatments ($P < .01$) (Figs. 1–4). The inverse relationship between the LDH and the MTS responses adds credence to the accuracy of the data. In the LDH assay, as the concentration of the drugs increased, cells became progressively more cytotoxic, leading to a greater absorbance reading in the LDH assay and a decrease in absorbance in the MTS assay with a concurrent decrease in percentage of viable cells.

For comparative studies, normal (non-cancerous) cells were subjected to identical treatments and bioassay analysis. The results obtained demonstrated the MCF-7 cells to exhibit significantly cytotoxicity in response to both pomegranate extracts and genistein when compared with the normal cells (Fig. 5).

Pomegranate extracts and genistein induce apoptosis in human MCF-7 breast cancer cells

Extensive cell death was observed in proliferating human breast cancer cells after treatments with pomegranate extracts and the isoflavone genistein. To determine if the treatment-induced cell death occurred through cytotoxic necrosis and/or apoptosis, cells were harvested and assayed for apoptosis induction with acridine orange/ethidium bromide and TUNEL. The results showed a significant amount of apoptotic cell death in the MCF-7 cells when compared with the normal cells (Fig. 6). The TUNEL assay showed the typical hallmark morphologic changes associated with apoptosis—which include chromatin condensation, protein breakdown, and DNA fragmentation in the treated cells.

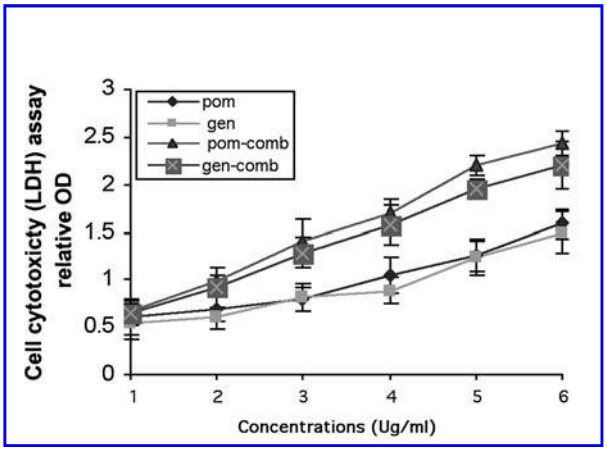

FIG. 1. Cytotoxic effect of pomegranate (pom) extracts and genistein (gen) [single and combination (comb) treatments for 24 hours] in human breast cancer (MCF-7) cells. MCF-7 cells were cultured and treated with different concentrations of pomegranate extracts alone ($P_{12–80}$) and in combination (P_{40}–$G_{10–70}$) and genistein alone ($G_{10–70}$) and in combination (G_{20}–$P_{12–80}$) for 24 hours. Then, LDH analysis was performed on the supernatants (see Materials and Methods). Concentrations are given on the *x*-axis: 1 = 0; 2 = P_{12} or G_{10} or P_{40}/G_{10} or G_{20}/P_{12}; 3 = P_{28} or G_{20} or P_{40}/G_{20} or G_{20}/P_{28}; 4 = P_{40} or G_{30} or P_{40}/G_{30} or G_{20}/P_{40}; 5 = P_{60} or G_{50} or P_{40}/G_{50} or G_{20}/P_{60}; 6 = P_{80} or G_{70} or P_{40}/G_{70} or G_{20}/P_{80}. Results were obtained from three independent experiments and expressed as mean ± standard error ($n = 3$). $P <$ .05 versus control. OD, optical density.

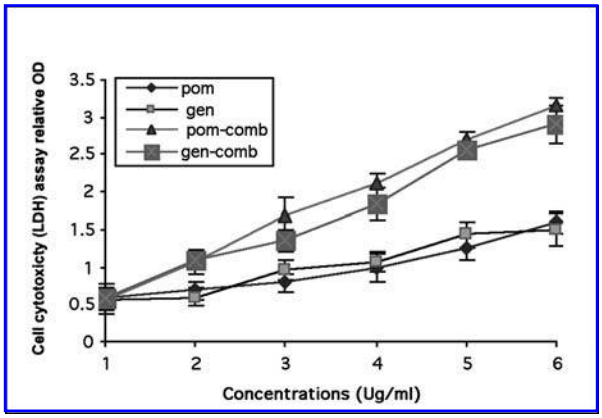

FIG. 2. Cytotoxic effect of pomegranate (pom) extracts and genistein (gen) [single and combination (comb) treatments for 72 hours] in human breast cancer (MCF-7) cells. MCF-7 cells were cultured and treated with different concentrations of pomegranate extracts alone ($P_{12–80}$) and in combination (P_{40}–$G_{10–70}$) and genistein alone ($G_{10–70}$) and in combination (G_{20}–$P_{12–80}$) for 72 hours. Then, LDH analysis was performed on the supernatants (see Materials and Methods). Concentrations are given on the *x*-axis: 1 = 0; 2 = P_{12} or G_{10} or P_{40}/G_{10} or G_{20}/P_{12}; 3 = P_{28} or G_{20} or P_{40}/G_{20} or G_{20}/P_{28}; 4 = P_{40} or G_{30} or P_{40}/G_{30} or G_{20}/P_{40}; 5 = P_{60} or G_{50} or P_{40}/G_{50} or G_{20}/P_{60}; 6 = P_{80} or G_{70} or P_{40}/G_{70} or G_{20}/P_{80}. Results were obtained from three independent experiments and expressed as mean ± standard error ($n = 3$). $P <$ .05 versus control. OD, optical density.

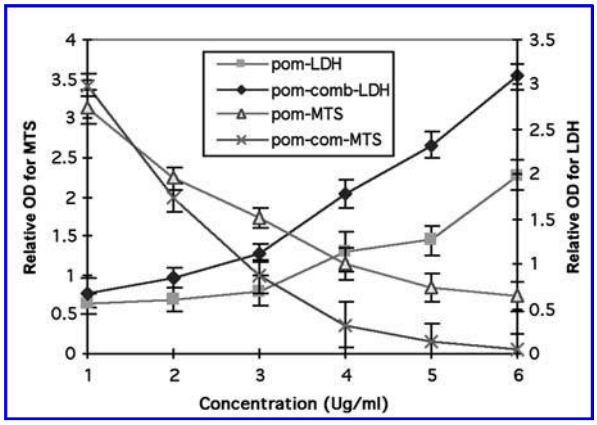

FIG. 3. Comparison of cell survival and cytotoxicity levels in single and combination [com(b)] treatments of pomegranate (pom) extracts (P) on human breast cancer (MCF-7) cells. MCF-7 cells were seeded and cocultured with $P_{12–80}$ and P_{40} + genistein ($G_{10–70}$) as described in Materials and Methods. After a 72-hour incubation, cells were subjected to MTS tetrazolium and LDH cytotoxicity assays. Note the correlation between MTS and LDH. Concentrations are given on the *x*-axis: 1 = 0; 2 = P_{12} or P_{40}/G_{10}; 3 = P_{28} or G_{20} or P_{40}/G_{20}; 4 = P_{40} or G_{30} or P_{40}/G_{30}; 5 = P_{60} or G_{50} or P_{40}/G_{50}; 6 = P_{80} or G_{70} or P_{40}/G_{70}. Results were obtained from three independent experiments and expressed as mean ± standard error ($n = 3$). $P <$ .05 versus control. OD, optical density.

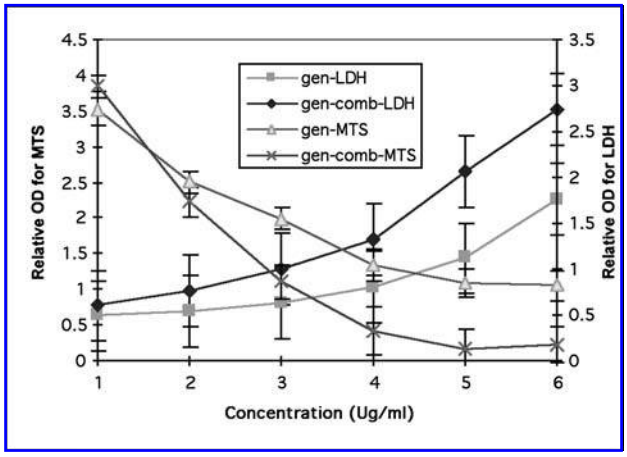

FIG. 4. Comparison of cell survival and cytotoxicity levels in single and combination (comb) treatments of genistein (gen) (G) on human breast cancer (MCF-7) cells. MCF-7 cells were seeded and cocultured with $G_{10–70}$ and G_{20} + pomegranate extracts ($P_{12–80}$) as described in Materials and Methods. After a 72-hour incubation, cells were subjected to MTS tetrazolium and LDH cytotoxicity assays. Note the correlation between MTS and LDH. Concentrations are given on the *x*-axis: 1 = 0; 2 = G_{10} or G_{20}/P_{12}; 3 = G_{20} or G_{20}/P_{28}; 4 = G_{30} or G_{20}/P_{40}; 5 = G_{50} or G_{20}/P_{60}; 6 = G_{70} or G_{20}/P_{80}. Results were obtained from three independent experiments and expressed as mean ± standard error ($n = 3$). $P <$ .05 versus control. OD, optical density.

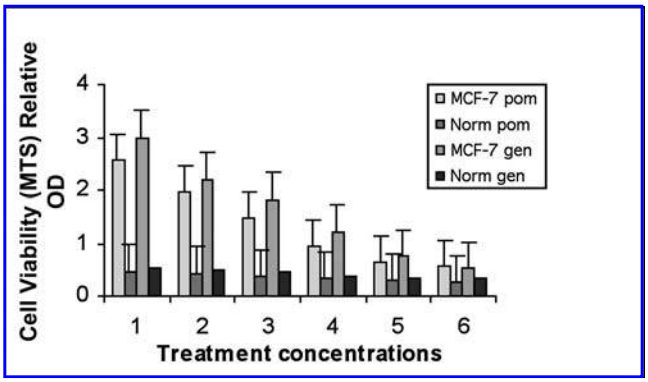

FIG. 5. Comparison of cell survival levels in single and combination treatments of human breast cancer (MCF-7) and normal (Norm) cells. MCF-7 and normal cells were seeded and co-cultured with pomegranate (pom) extracts ($P_{12\text{-}80}$) and genistein (gen) ($G_{10\text{–}70}$) as described in Materials and Methods. After a 24-hour incubation, cells were subjected to MTS assay. Note the differences between the extremes of the two types of cells. Concentrations are given on the *x*-axis): 1 = 0; 2 = P_{12} or G_{10}; 3 = P_{28} or G_{20}; 4 = P_{40} or G_{30}; 5 = P_{60} or G_{50}; 6 = P_{80} or G_{70}. Results were obtained from three independent experiments and expressed as mean ± standard error ($n = 3$). $P < .05$ versus control. OD, optical density.

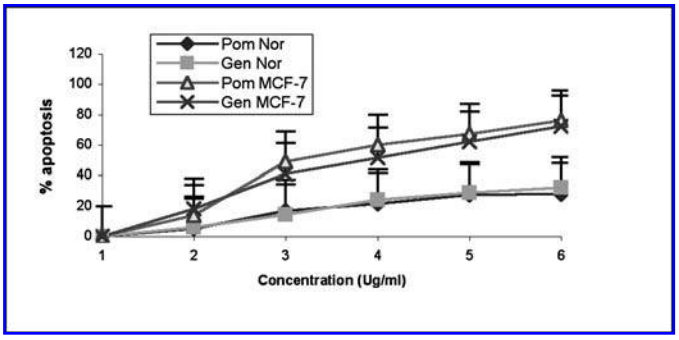

FIG. 6. Comparison of apoptosis detection in normal (Nor) and human breast cancer (MCF-7) cells treated with various levels of single and combination treatments with pomegranate (Pom) extracts and genistein (Gen). Apoptosis was measured with staining with acridine orange/ethidium bromide. MCF-7 and normal cells were seeded and cocultured with Pom ($P_{12\text{–}80}$) and Gen ($G_{10\text{–}70}$) as described in Materials and Methods. After a 24-hour incubation, cells were subjected to MTS assay. Note the differences between the extremes of the two types of cells. Concentrations are given on the *x*-axis: 1 = 0; 2 = P_{12} or G_{10}; 3 = P_{28} or G_{20}; 4 = P_{40} or G_{30}; 5 = P_{60} or G_{50}; 6 = P_{80} or G_{70}. Results were obtained from three independent experiments and expressed as mean ± standard error ($n = 3$). $P < .05$ versus control. OD, optical density.

These changes were especially pronounced in the combination treatments compared with the single treatments ($P < .01$).

DISCUSSION

The present study was designed to investigate the potential therapeutic capabilities of pomegranate extracts and genistein in human MCF-7 breast cancer cells. The results indicate that both pomegranate extracts and genistein have significant *in vitro* inhibitory effect on the growth rate of MCF-7 breast cancer cells. The results also demonstrate that both pomegranate and genistein, in single and combination treatments, induced apoptosis in the cancer cells in a time- and dose-dependent manner. Growth inhibition effects of both pomegranate and genistein have been observed in many human adenocarcinoma cell lines.[7,8,38] The growth inhibition correlated significantly with the degree of treatment-induced cell death and induction of apoptosis in the target cells. This is consistent with previous reports.[38] The treatment-induced morphological changes in the cells (indicative of cell differentiation, inhibition, and cell death) were consistent with previously observed results.[36] The significant correlation between the two nonradioactive assays (LDH and MTS) augments the validity of the results.

In preliminary studies in our laboratory, pomegranate extracts and genistein were found to induce apoptosis in about 50% of the cells at concentrations of 40 μg/mL and 20 μg/mL, respectively. These doses were used in the present study to make the combination treatments, where P_{40} was used as the base for the pomegranate combinations and G_{20} as the base for the genistein combinations. For both phytochemicals, the combination treatments were found to have greater effects on the cells. These results revealed that both pomegranate extracts and genistein, in single and combination treatments, induced apoptosis as the main mechanism of cell death and growth inhibition in the target cells. The results are consistent with previous studies.[30,39,40]

Apoptosis, identified as one of the most fundamental biological processes in eukaryotes in which individual cells die by activating intrinsic "suicide" mechanisms, has been suggested to play a key role in damage to cells, caused by a variety of insults.[41] Previous studies demonstrated that both genistein extracts and pomegranate caused DNA strand breakage in tumor cells[42–44] and apoptotic cell death in several tumor cells.[43,45,46] Genistein itself has been found to arrest cell cycle progression.[44,45] Many cancer chemotherapeutic drugs with DNA damage capability are known to induce accumulation of p53 in the cells, indicating cell cycle arrest or programmed cell death (apoptosis).[47,48] The fact that pomegranate extracts and genistein induce apoptosis in MCF-7 cells, as demonstrated here, justifies the inclusion of both phytochemicals in diagnostic and therapeutic strategies in dealing with human pathology. Given the results obtained in this study and those from previous experiments, one can speculate that there are substantial possibilities for using both pomegranate extracts and genistein as potential tools in human pathophysiology research, especially breast cancer.

It was concluded that: (1) pomegranate has an inhibitory effect on MCF-7 breast cancer cells, (2) growth and proliferation inhibition is through induction of apoptosis, (3) combination treatment is significantly more effective than single treatment with either drug alone, and (4) apoptosis induction is both dose- and time-dependent. These results offer justification for further studies on the anticancer activities of these test drugs.

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