Pomegranate Extract, A Prooxidant with Antiproliferative and Proapoptotic Activities Preferentially Towards Carcinoma Cells

Jeffrey H. Weisburg, Alyssa G. Schuck, Malki S. Silverman, Channa G. Ovits-Levy, Loriel J. Solodokin, Harriet L. Zuckerbraun and Harvey Babich*

Department of Biology, Stern College for Women, Yeshiva University, 245 Lexington Avenue, New York NY 10016, USA

Abstract: The antiproliferative and proapoptotic effects of pomegranate extract (PE), as correlated with its prooxidant activity, were studied. PE exerted greater antiproliferative effects towards cancer, than to normal, cells, isolated from the human oral cavity. In cell-free systems, PE generated hydrogen peroxide (H_2O_2) in cell culture media and in phosphate buffered saline, with prooxidant activity increasing from acidic to alkaline pH, and oxidized glutathione (GSH) in an alkaline, phosphate buffer. Detection of PE-generated H_2O_2 was greatly lessened in medium amended with *N*-acetyl-L-cysteine. Using HSC-2 carcinoma cells as the bioindicator, the cytotoxicity of PE was potentiated towards cells pretreated with the GSH depleter, 1-chloro-2,4-dinitrobenzene, and attenuated in cells cotreated with the H_2O_2 scavengers, catalase, pyruvate, and divalent cobalt ion. Intracellular GSH was lessened in cells treated with PE; GSH depletion in PE-treated cells was confirmed visually with the fluorescent dye, Cell Tracker™ Green 5-chloromethylfluorescein diacetate. These studies demonstrated that the antiproliferative mechanism of PE was, in part, by induction of oxidative stress. The mode of cell death was by apoptosis, as shown by flow cytometry, activation of casapase-3, and cleavage of PARP. Lessening of caspase-3 activation and of PARP cleavage in cells cotreated with PE and either cobalt or pyruvate, respectively, as compared to PE alone, indicated that apoptosis was through the prooxidant nature of PE.

Keywords: Pomegranate, prooxidant, oxidative stress, apoptosis, polyphenols, nutraceuticals.

1. INTRODUCTION

Epidemiologic data, coupled with studies using laboratory animal models, have indicated that 30% of all cancer cases are linked to poor dietary habits, with this value increasing to 70% for cancers of the gastrointestinal tract. A complimentary body of literature has linked the consumption of fruits and vegetables to a substantial reduction in the risk of developing cancers. Carcinogenesis is a gradual process during which precancerous cells accumulate mutations in genes involved in cell proliferation, resistance to apoptosis, and induction of angiogenesis needed for metastasis. Chemoprevention through consumption of fruits and vegetables involves the intake of high levels of phytochemicals which potentially can reverse, suppress, or prevent progression to invasive cancer. The spectrum of phytochemicals with potentially anticarcinogenic activities includes polyphenols, carotenoids, and organosulfur compounds [1].

The research presented herein evaluated the antiproliferative and proapoptotic effects of a pomegranate polyphenol-enriched extract (PE) to cancer cells derived from tissues of the human oral cavity. The incidence of squamous cell carcinoma of the head and neck is approximately 43,000 annually in the United States, with the five-year survival rate of 40%. Patients who develop this disease usually have a history of smoking and alcohol abuse, with the oral cavity being one of the most common sites for tumor initiation [2]. As such, the consumption of foods containing high levels of chemopreventive phytochemicals is a potentially attractive treatment for premalignant lesions of the oral cavity. In clinical studies, Halder *et al.* [3], for the consumption of black tea, and Li *et al.* [4], for the intake of green tea, used this approach to control oral precancerous lesions, primarily leukoplakia, often associated with oral carcinoma.

The health benefits of pomegranates have focused on their antioxidant properties, attributed to their high content of soluble polyphenols, including the hydrolysable ellagitannin, punicalagin, the most abundant polyphenol and accounting for >50% of the antioxidant activity of pomegranate [5]. Clinical studies on the consumption of pomegranate juice have indicated cardiovascular protective effects, including lowered blood pressure, reduced oxidation of low-density lipoproteins [6], improvement in blood flow [7], and a reduction in plasma thiobarbituric reactive substances (TBARS), which are linked to cardiovascular disease [8]. A phase II clinical trial of pomegranate juice consumption in patients with prostate cancer reported a significant prolongation of the doubling time for prostate specific antigen [9]. Studies in laboratory animal models demonstrated that pomegranate fruit extracts and/or diets supplemented with pomegranate juice were inhibitory to tumor growth [10], had neuroprotective effects against neonatal brain injury from reduced blood flow and oxygen [11], and reduced inflammation [12]. Mechanistic studies conducted with cell culture have complemented the findings cited above, e.g., the growth inhibitory and apoptosis-inducing effects on cancer cells [5, 13] and the inhibition of cellular anti-inflammatory activities [14] of pomegranate extract. Research on the potential health effects of the consumption of pomegranate juice to the oral cavity has focused on its antimicrobial activity [15].

Polyphenols exhibit both antioxidant and prooxidant activities [16] and there is an increasing body of evidence, conducted primarily from cell culture studies, to indicate that the prooxidant activities of plant-derived polyphenols were at levels sufficient to induce oxidative stress, which accounted for their antiproliferative and proapoptotic activities. Such plant-derived polyphenols with prooxidant activities included curcumin [17], ginkgetin [18], epigallocatechin gallate [19, 20], theaflavin-3,3'-digallate [21], and gallic acid [22]. Plant extracts from *Ginkgo biloba* [23], green tea, and black tea [20] also exhibited prooxidant activities that were correlated with their antiproliferative and proapoptotic effects. Due in part to oncogenic stimulation, mitochondrial malfunction, and increased metabolic activity, cancer cells exhibit intrinsic hypersensitivity to oxidative stress [24]. Many studies have also shown the greater sensitivities of cancer, than of normal, cells to the prooxidant activities of plant-derived polyphenols, thus, suggesting their potential usage as chemopreventive agents [25-27].

The prooxidant activity of pomegranate extract has not, as yet, been evaluated. The high polyphenol content in pomegranate, suggest that it may be a strong generator of reactive oxygen species (ROS). The aims of this research were: (a) to evaluate the prooxidant nature of a pomegranate polyphenol-enriched extract (PE) and

*Address correspondence to these authors at the Department of Biology, Stern College for Women, Yeshiva University, 245 Lexington Avenue, New York, NY USA; Tel.: +1 212 340 7739;
Fax: +1 212 340 7883; E-mail: babich@yu.edu

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(b) to determine whether the generation of ROS was sufficient to inhibit cell growth and to induce apoptotic cell death. Human HSC-2 carcinoma cells were used as the target bioindicators. These cells, derived from the human oral cavity, were extensively employed in prior research to evaluate the little studied, prooxidant nature of plant-derived polyphenols. The research presented herein is the initial study evaluating the prooxidant nature of a pomegranate polyphenol extract and relating this prooxidant activity to biological effects, i.e., the induction of oxidative stress leading to growth inhibition and to cell death via apoptosis.

2. MATERIALS AND METHODS

2.1 Cell Culture

Human squamous carcinoma HSC-2 cells derived from the floor of the oral cavity were obtained from H. Sakagami, Department of Dental Pharmacy, Meikai University School of Dentistry, Saitama, Japan, human tongue squamous carcinoma CAL27 cells from D.A. Tipton, University of Tennessee, College of Dentistry, Memphis, TN, USA, human squamous carcinoma SCC 1483 cells, developed from a retromolar trigone, from Y. Ramanathan, Head and Neck Service, Memorial Sloan-Kettering Cancer Center, New York, NY, USA, and human normal gingival HF-1 fibroblasts from P. Sacks, New York University School of Dentistry, New York, NY, USA. Growth medium consisted of Dulbecco's modified Eagle's medium (DMEM with pyruvate (Cat. No.11995-080); Invitrogen, Carlsbad, CA, USA), supplemented with 10% fetal bovine serum (FBS), 50 U/ml penicillin G, 50 µg/ml streptomycin sulfate, and 1.25 µg/ml amphotericin B. Cultures were maintained in a humidified atmosphere with 5.5% CO_2 at 37˚C. Cell dissociation was achieved with 0.1% trypsin-0.04% EDTA.

2.2. Chemicals

The pomegranate extract (PE), a purified natural pomegranate ellagitannin-enriched polyphenol extract (designated POMx and obtained as a gift from Pom Wonderful, LLC, Los Angeles, CA, USA), was solubilized in 25% dimethyl sulfoxide (DMSO). PE has a polyphenol content of 61% gallic acid equivalents, consisting of oligomers of 2-10 repeating units of gallic acid, ellagic acid, and glucose in different combinations (77%), ellagitannins as punicalagins and punicalins (19%), and free ellagic acid (4%) [8]. PE is standardized to an ellagitannin content of 37% punicalagin [5].

A stock solution of $CoCl_2.6H_2O$ (Aldon Corp., Avon, NY, USA) was prepared in water and of 1-chloro-2,4-dinitrobenzene (CDNB) (Sigma Chemical Co., St. Louis, MO, USA) in ethanol. Immediately before use, catalase (bovine liver) (Sigma) was introduced directly into exposure medium consisting of DMEM (without pyruvate, Cat. No.11995-065, Invitrogen), 10% Serum Plus (JRH Biosciences, Lenexa, KS, USA), 2% FBS, and antibiotics. Studies with pyruvate utilized commercially-available DMEM with 110 mg/L pyruvate (Cat. No.11995-080; Invitrogen). Stock concentrations of the test agents were made at high enough concentrations so that when diluted prior to use, the residual solubilizing concentrations of ethanol (0.5% and less) and DMSO (0.1% and less) were not cytotoxic.

2.3. Cell Proliferation Assay

Individual wells of a 96-well microtiter tissue culture plate were inoculated with 0.2 ml of the growth medium containing 2 x 10^4 cells/well for the carcinoma cells and 1.5 x 10^4 cells/well for the fibroblasts. After a 24-hr incubation, the growth medium was removed and replaced with exposure medium, with or without varied concentrations of PE. In some studies, the cells were either coexposed to PE with 100 Units/ml catalase, 0.25 mM Co^{2+}, or 110 mg/L (~ 1 mM) pyruvate or preexposed for 20 min to 25 µM CDNB (in PBS), followed by treatment with the extract. One complete row, i.e., 8 wells, was used for each concentration of test agent, with and without the accompanying variable. After exposure to the test agents, viability was assessed with the neutral red (NR) assay, which is based on the uptake and accumulation of the supravital dye, NR [28].

The protocol for the NR assay was as follows. A foil-wrapped 4 mg/ml aqueous stock suspension of NR was stored at room temperature. The stock was diluted to a working concentration of 0.04 mg/ml NR in exposure medium and incubated overnight at 37˚C. After a 24-hr exposure of the cells to the test agents, the medium was removed, 0.2 ml of NR-containing medium was added per well, and incubation was continued for 1 hr at 37˚C. Cells were then rapidly washed and fixed with a 0.2 ml solution of 0.5% formalin-1% $CaCl_2$ (vol/vol) and the NR incorporated into the viable cells was released into the supernatant with 0.2 ml solution of 1% acetic acid-50% ethanol. Absorbance was recorded at 540 nm with a microtiter plate spectrophotometer.

2.4. Intracellular Reduced Glutathione (GSH) Assay

Measurements of intracellular GSH were performed according to the procedures of Weisburg *et al.* [20]. HSC-2 cells, maintained in growth medium and grown to confluence in 60 mm tissue culture plates, were washed with PBS and treated for 4 hr with serum-free DMEM (without pyruvate) unamended and amended with concentrations from 100 to 500 µg/ml PE. Three plates were used per concentration of extract. Thereafter, the cells were washed with PBS, lysed with 0.06 ml of 0.2% Triton X-100, and proteins were precipitated with 0.06 ml of 5.0% sulfosalicyclic acid. Cells, harvested by scraping, were centrifuged at 12,000 x g for 5 min. The GSH concentration was determined in 0.1 ml aliquots of the acid-soluble extract by measuring the oxidation of GSH with a 6 mM solution of 5,5'-dithiobis(2-nitrobenzoic acid), prepared in a phosphate buffer/EDTA (pH 7.5), to glutathione disulfide (GSSG), with a stoichiometric formation of 5-thio-2-nitrobenzoic acid, a yellow chromagen measured spectrophotometrically at 412 nm. With each assay, a standard curve was generated with known amounts of GSH and the data were recorded as nmoles intracellular GSH/10^6 cells.

2.5. Cell-free Assay for Authentic GSH

The reactivity of PE with GSH was assayed in a cell-free system. Solutions of phosphate buffer/EDTA (pH 7.5), unamended and amended with 100 nmoles/ml GSH and with 0 to 500 µg/ml PE, were incubated at room temperature for 1 hr. Thereafter, 0.5 ml aliquots were removed and assayed for GSH as above [23].

2.6. Hydrogen Peroxide Assay

Measurement of hydrogen peroxide (H_2O_2) production was carried out by the ferrous ion oxidation xylenol orange (FOX) method using the PeroXOquant Quantitative Peroxide Assay Kit, lipid-soluble formulation (Pierce Biotechnology, Rockford, IL, USA). The protocol was followed as suggested by the manufacturer, but with some modifications. Briefly, 90 µl samples of serum-free DMEM, with and without pyruvate, of serum-free Minimum Essential Medium (MEM), RPMI 1640 medium, and McCoy's 5A medium (all lacking pyruvate), and of PBS, adjusted to pH 5.4, 6.4, and 7.4, were amended with PE, mixed with 10 µl of methanol, and incubated at room temperature for up to 2 hr. In other experiments, the generation of H_2O_2 was studied in PE-amended exposure medium (DMEM + 2% FBS + 10% Serum Plus) and in DMEM supplemented with varying concentrations of the antioxidant, *N*-acetyl-L-cysteine. Incubations were at room temperature and after designated times the FOX reagent (0.9 ml) was added, followed by vortexing and a 30-min incubation at room temperature. The solutions were centrifuged at 12,000 x g for 10 min and absorbance of the supernatant was read at 580 nm against a blank consisting of 90 µl of exposure medium or PBS, 10 µl of methanol, and 0.9 ml of FOX reagent. A standard curve was generated using H_2O_2 as the reference peroxide [29].

2.7. Apoptosis Assay: Flow Cytometry

HSC-2 cells, grown to ~80% confluence in 60 mm plates, were untreated or treated for 20 hr with varying concentrations of PE.

Afterwards, the cells were washed with PBS, typsinized, and diluted to a concentration just under 5 x 10^5 cells/ml. A 20 ml sample of cells, added to 380 ml of Guava ViaCount Reagent (Millipore, Billierica, MA, USA), was placed on ice and in the dark for 5 min. Cell viability, apoptosis, and cell death were determined with a Guava Easycyte Miniflow Cycytometer (Millipore). The Guava Viacount Reagent distinguishes between viable, apoptotic, and non-viable cells based on the differential permeability of its DNA-binding dyes. The fluorescence of each dye is resolved operationally to allow for the quantitative assessment of viable, apoptotic, and non-viable cells present in a suspension.

2.8. Apoptosis Assay: Western Blotting

HSC-2 cells grown to ~80% confluence were untreated or treated for 20 hr with PE in exposure medium, without and with pyruvate. Cells lysed with RIPA buffer (Thermo Scientific, Rockford, IL, USA), containing complete EDTA-free protease inhibitor (Roche Diagnostics Corp., Indianapolis, IN, USA), were then centrifuged (12, 000 x g, 10 min) to remove cellular debris. Protein concentrations of the cell lysates were quantified with the BCA Protein Assay Kit (Pierce Biotechnology, Rockford, IL, USA). Equal concentrations of total protein from each sample were separated by SDS-PAGE (10% for PARP detection; 4-20% for caspase-3 detection) (Thermo Scientific), electroblotted to nitrocellulose membranes (Amersham Biosciences, Piscataway, NJ, USA), and blocked with 5% dry milk in Tris-buffered saline containing 0.1% Tween 20. Membranes were then probed with polyclonal anti-poly(ADP-ribose)-polymerase (anti-PARP) antibody (rabbit antiserum, diluted 1:5,000 (Abcam, Cambridge, MA, USA)) to detect both full-length and cleaved PARP, or with anti-caspase-3 (rabbit antiserum, Cell Signaling Technology, Danvers, MA, USA). The portion of the membrane corresponding to the molecular weight of actin was probed with a rabbit anti-actin antibody (rabbit antiserum, diluted 1:7,500 (Santa-Cruz Biotechnology, Inc., Santa Cruz, CA, USA)) to ensure equal protein loading. Following incubation with the appropriate peroxidase-conjugated secondary antibodies (1:5,000 dilution), membranes were developed using the ECL detection kit (Amersham). Western blot analyses were performed a minimum of three times [21].

2.9. Fluorescent Detection of Intracellular Thiols

HSC-2 cells, grown on coverslips in 35 mm cell culture plates with complete medium, were then exposed to PE in serum-free DMEM (without pyruvate) for 4 hr. The fluorescent dye, Cell Tracker™ Green CMFDA (Invitrogen), dissolved in DMSO to a 10 mM stock solution, was diluted to 5 µM in warm serum-free DMEM without pyruvate and then added for 40 minutes at 37ºC. The dye was removed and replaced with warm serum-free DMEM without pyruvate for an additional 30 minutes at 37ºC to activate the dye. The media were removed and the cells washed with PBS and fixed with 3.7% formaldehyde in PBS for 15 min at room temperature [29].

2.10. Statistics

All experiments were performed a minimum of three times. Cytotoxicity graphic data were presented as the mean percentages of control ± standard errors of the mean (S.E.M.). Experimental data were analyzed with a one-way analysis of variance (ANOVA) followed by Tukey's multiple range test for significant differences. The *P* value of the effect had to be ≤0.05 to be considered significant.

3. RESULTS

3.1. Antiproliferative Effects of a 24-hr Exposure to PE

Concentration-response dependent cytotoxicity curves for a 24-hr exposure of the carcinoma cell lines and of the normal fibroblasts to PE are presented in Fig. (**1**). The responses of the three carcinoma cell lines clustered together. Toxicity was noted initially at 50 µg/ml PE for the CAL27 cells ($P \leq 0.05$), at 75 µg/ml PE for the SCC1483 ($P \leq 0.05$) and HSC-2 ($P \leq 0.01$) cells, and at 125 µg/ml PE for the HF-1 fibroblasts ($P \leq 0.01$). Midpoint cytotoxicity (NR_{50}) values were approximated at 100 µg/ml PE for the HSC-2 and CAL 27 cells, at 125 µg/ml PE for the SCC1483 cells, and at 200 µg/ml PE for the HF-1 fibroblasts.

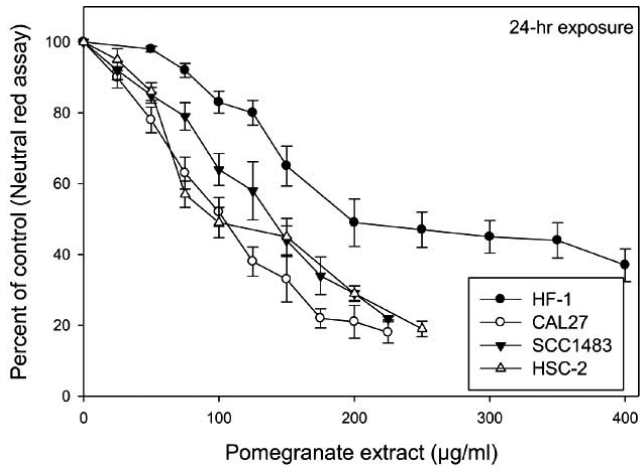

Fig. (1). Proliferation of carcinoma HSC-2 (n= 10), CAL27 (n = 5), and SCC1483 (n = 6) cells and normal gingival HF-1 fibroblasts (n = 8) after a 24-hr exposure to pomegranate extract, as determined with the neutral red (NR) assay. The data are expressed as the arithmetic mean percent of control ± S.E.M.

3.2. Prooxidant Nature of PE

The generation of H_2O_2, as quantified with the FOX assay, was determined in various cell culture media and in PBS, adjusted to different pHs. In serum-free DMEM, without pyruvate, the generation of H_2O_2 was both time-dependent and PE concentration-dependent. Increasing the concentration of PE from 50 to 250 µg/ml and the incubation time from 1- to 2-hr, progressively increased the generation of H_2O_2 (Fig. (**2**)). The level of H_2O_2 generated after a 2-hr incubation varied in different media, all lacking pyruvate and all without serum added. The trend of increasing H_2O_2 in PE-amended media followed the sequence DMEM > MEM > RPMI > McCoy's. A much significantly reduced level of H_2O_2 was noted in commercially-available DMEM containing pyruvate, a scavenger of H_2O_2. In PBS amended with 250 µg/ml PE, increasing

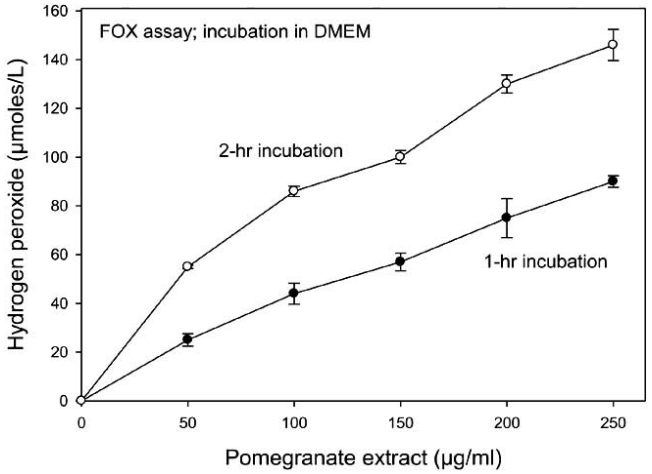

Fig. (2). Generation of hydrogen peroxide (H_2O_2), as determined by the FOX assay, in Dulbecco's modified Eagle medium (DMEM), amended with varying concentrations of pomegranate extract over a 1- and a 2-hr incubation. The data are expressed as the arithmetic mean ± S.E.M.; n = 3.

the pH from 5.4 to 6.4 and then to 7.4, resulted in greater generation of H_2O_2. At a comparable PE concentration (i.e., 250 µg/ml) and incubation time (i.e., 2 hr), approximately 50% less H_2O_2 was detected in alkaline PBS than in DMEM without pyruvate (Fig. (**2**)); Table **1**).

Table 1. Generation of Hydrogen Peroxide (H_2O_2) by 250 µg/ml Pomegranate Extract after a 2-hr Incubation in Different Commercially-Available Cell Culture Media and in Phosphate Buffer Saline (PBS) Maintained at Different pHs

Test System	H_2O_2 (µmoles/L)
Medium	
DMEM	120 ± 6.9
MEM	89 ± 7.5
RPMI	80 ± 8.3*
McCoy's	66 ± 2.9**
DMEM (with pyruvate)	3 ± 2.0**
PBS	
5.4	7 ± 3.4**
6.4	14 ± 1.1**
7.4	49 ± 4.1

H_2O_2 was determined with the FOX assay. The data are expressed as the arithmetic mean ± S.E.M. Asterisks indicate significant differences either between DMEM and the other cell culture media (all at approximately, pH 7.2) or between PBS at pH 7.4 and at pH 5.4 and 6.4. * = $P \leq 0.05$; ** = $P \leq 0.01$; n = 3.

Detection of H_2O_2 in exposure medium (DMEM + 2% FBS + 10% Serum Plus) amended with PE followed both a time- and a concentration-dependent pattern (Fig. (**3**)). Focusing on 250 µg/ml PE after a 2-hr incubation, the level of H_2O_2 detected in exposure medium (Fig. (**3**)) was intermediate between that for PE-amended DMEM and for PE-amended PBS, pH. 7.4 (Table **1**).

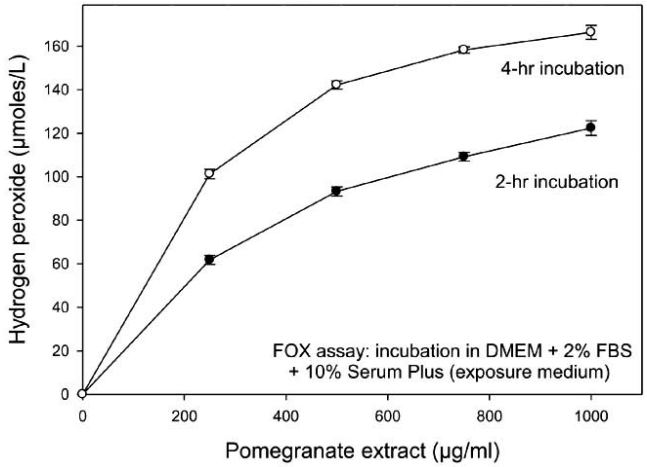

Fig. (3). Generation of hydrogen peroxide (H_2O_2), as determined by the FOX assay, in exposure medium (Dulbecco's modified Eagle medium (DMEM) supplemented with 2% fetal bovine serum (FBS) and 10% serum Plus; pH 7.2), amended with varying concentrations of pomegranate extract over a 2- and 4-hr incubation. The data are expressed as the arithmetic mean ± S.E.M.; n = 3.

Supplementation of *N*-acetyl-L-cysteine, a sulfhydryl-containing antioxidant, into DMEM amended with 250 µg/ml PE, reduced the level of detectable H_2O_2, both in a concentration- and time-pattern. Addition of *N*-acetyl-L-cysteine at 2.5 mM greatly lessened, and from 5 to 10 mM almost completely eliminated, the detection of H_2O_2 in PE-amended DMEM (Fig. (**4**)).

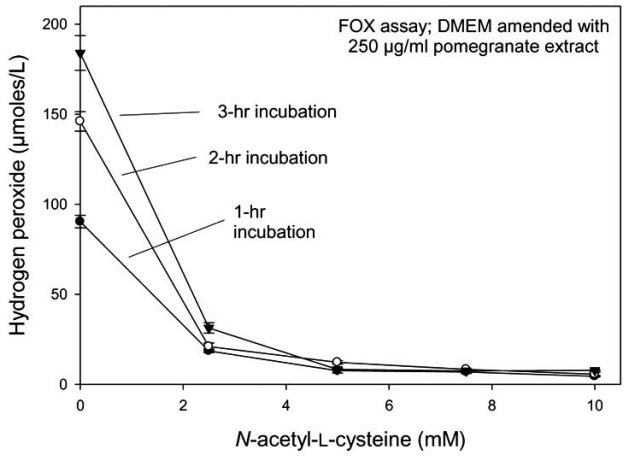

Fig. (4). Generation of hydrogen peroxide (H_2O_2), as determined by the FOX assay, in Dulbecco's modified Eagle medium (DMEM), pH 7.2, amended with 250 µg/ml pomegranate extract and supplemented with varying concentrations of *N*-acetyl-L-cysteine, over a 1-,2-, and 3-hr incubation. The data are expressed as the arithmetic mean ± S.E.M.; n = 3.

3.3. Cytotoxicity of PE as Mediated by Scavengers of H_2O_2

As the generation of H_2O_2 by PE suggested that its mode of cytotoxicity may be due, in part, to the induction of oxidative stress, attention was focused on discerning the interaction between PE and scavengers of H_2O_2. Albeit via different mechanisms, catalase, divalent cobalt, and pyruvate all scavenge H_2O_2. The 24-hr cytotoxicities of 200 and 250 µg/ml PE towards the HSC-2 cells were lessened, although not completely eliminated, in the presence of 110 mg/L pyruvate, 100 Units/ml catalase, and 250 µM Co^{2+} (Fig. (**5**)).

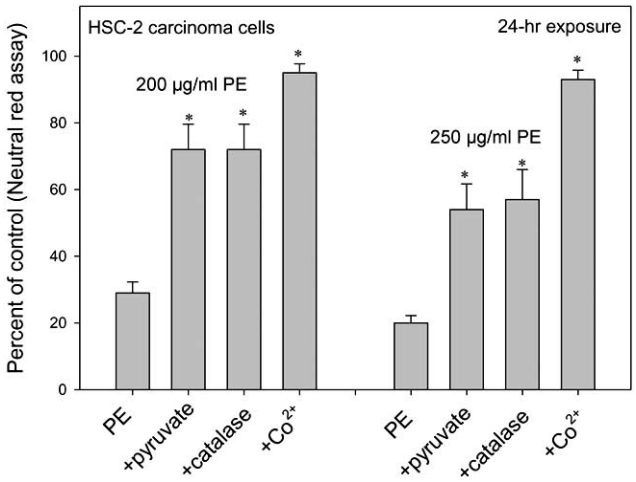

Fig. (5). Lessening of the cytotoxicity of pomegranate extract to HSC-2 cells upon coexposure with either 110 mg/L pyruvate, 100 Units/ml catalase, or 0.25 mM $CoCl_2.6H_2O$. Cytotoxicity was determined with the neutral red (NR) assay after a 24-hr exposure. The data are expressed as the arithmetic mean percent of the relative control ± S.E.M., were computed for each specific hydrogen peroxide scavenger + pomegranate extract as compared to pomegranate extract alone. *$P \leq 0.01$ as compared to pomegranate extract alone; n = 3 - 4.

3.4. PE as an Inducer of Oxidative Stress and the Role of GSH

Reduced GSH, a thiol-containing tripeptide, is the main intracellular antioxidant in the cell's repertoire against oxidative defense. Initial studies in a cell-free system using authentic GSH showed that in the presence of increasing PE concentrations, the level of GSH was progressively and quickly depleted (Table **2**).

These data suggested that the H_2O_2 generated by PE was actively scavenged by GSH.

Table 2. Direct Interaction between Pomegranate Extract and Authentic Reduced Glutathione after a 1-hr Incubation in Phosphate Buffer/EDTA (pH 7.5)

Pomegranate Extract (µg/ml)	Glutathione (nmoles/L)
Untreated	50 ± 0.8
62.5	36 ± 0.9*
125	25 ± 1.1*
250	4 ± 1.3*
375	2 ± 0.8*
500	0 ± 0.0*

The data are expressed as the arithmetic mean ± S.E.M. *Asterisks indicate significant differences ($P \leq 0.01$) between the untreated control and the samples amended with pomegranate extract; n = 3.

Depletion of intracellular GSH was determined in HSC-2 cells after a 4-hr incubation to increasing levels of PE in serum-free DMEM (without pyruvate). Increasing the concentration from 100 to 250 and then to 500 µg/ml PE progressively lowered the intracellular concentration of GSH Fig. (**6A**), which may have been related to the increasing generation of H_2O_2. Depletion of intracellular GSH upon exposure to PE was confirmed by fluorescent staining of intracellular thiols using Cell-Tracker™ Green CMFDA. The fluorescence intensity of the dye is a function of the concentration of intracellular thiol-containing molecules. Control cells not exposed to PE stained bright green Fig. (**6B**), whereas little fluorescence was noted in HSC-2 cells exposed to PE Fig. (**6C**). To further study the role between intracellular GSH and the prooxidant nature of PE, HSC-2 cells were pretreated with CDNB, a GSH depleter, prior to their 24-hr exposure to PE. The cytotoxicity of PE to the HSC-2 cells was potentiated upon prior treatment with CDNB Fig. (**7**).

3.5. PE as an Inducer of Apoptosis

As oxidative stress is a known inducer of cell apoptosis, the generation of H_2O_2 by PE may be a factor in cell death via apoptosis. Flow cytometric analyses of HSC-2 cells untreated and treated with PE showed that as the concentration of PE increased, the number of viable cells decreased and the number both of apoptotic and non-viable cells increased Fig. (**8A, B**). These results were complemented with immunoblot analyses of specific apoptosis marker proteins. Caspase-3 is a key executioner of apoptotic cell death; its activation is indicated by cleavage of the pro-enzyme at aspartic acid 175, yielding 17/19 kD and 12 kD active products. Immunoblot analysis of cell lysates treated with PE showed active caspase proteins in cells treated with 200 µg/ml PE and 250 µg/ml PE. Cells treated with PE in the presence of 0.25 mM $CoCl_2$, however, showed no caspase activation, even at a concentration of 250 µg/ml PE Fig. (**9A**). Another marker of irreversible apoptotic cell death is the inactivation of poly(ADP-ribose) polymerase (PARP) through cleavage by caspases. Detection of PARP fragments is an indicator of apoptosis. Lysates from cells untreated (control) and treated with 100 to 250 µg/ml PE, in the absence and presence of 110 mg/L pyruvate were analyzed to detect full-length PARP proteins and its ~85 kD cleavage product. In pyruvate-free DMEM, cleaved PARP fragments occurred at 150 µg/ml PE and greatly

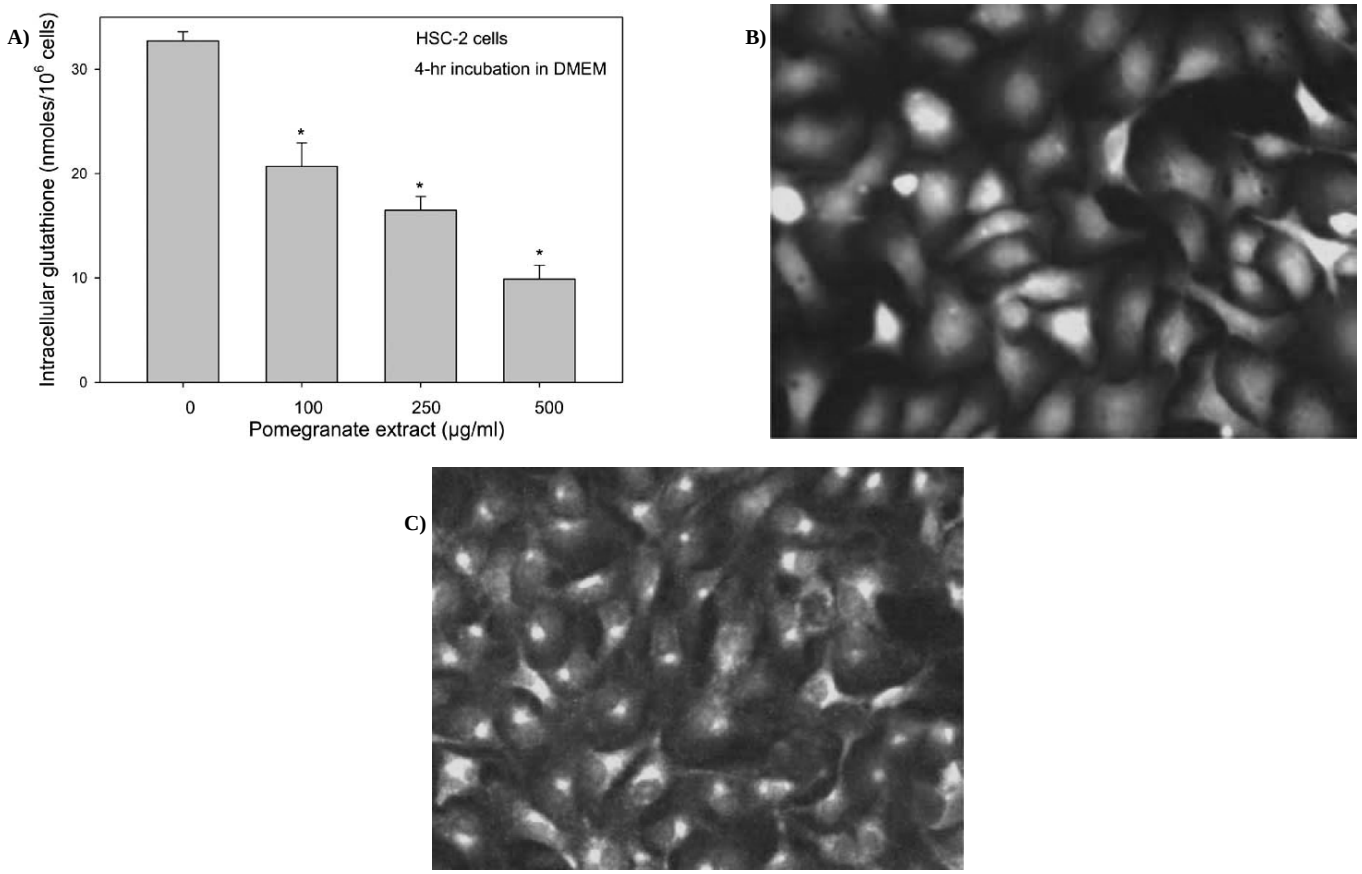

Fig. (6). (A) Lessening of the intracellular concentration of glutathione following a 4-hr incubation of HSC-2 cells in Dulbecco's modified Eagle medium (DMEM) lacking pyruvate. The data are expressed as the arithmetic mean ± S.E.M. *$P \leq 0.01$ as compared to untreated control; n = 5.**(B,C).** Depletion of intracellular glutathione, as detected by the fluorescent dye, Cell Tracker™ Green CMFDA. HSC-2 cells **(B)** untreated and **(C)** exposed for 4 hr to 250 µg/ml pomegranate extract in Dulbecco's modified Eagle medium lacking pyruvate. X320.

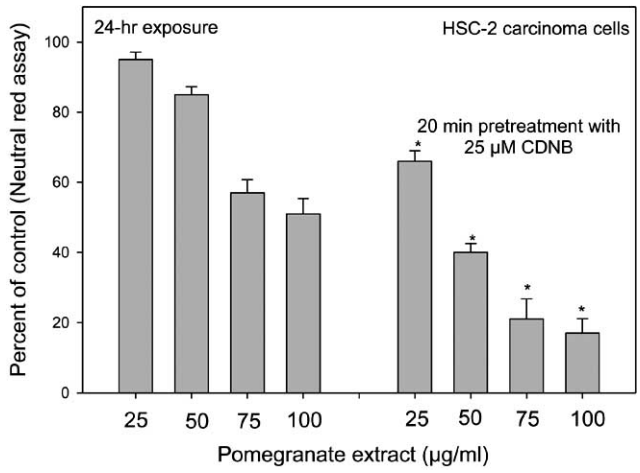

Fig. (7). Potentiation of the cytotoxicity of pomegranate extract to HSC-2 cells pretreated for 20 min to 25 μM 1-chloro-2,4-dinitrobenzene (CDNB) ; Cytotoxicity was determined with the neutral red (NR) assay after a 24-hr exposure. The data are expressed as the arithmetic mean percent of the relative control ± S.E.M. The toxicity of pomegranate extract was compared to untreated control cells and the toxicity of a CDNB preexposure + pomegranate extract was compared to cells preexposed to CDNB but not treated with pomegranate extract. *$P \leq 0.01$ as compared to pomegranate extract alone; n = 5.

increased as the concentration was increased to 200 and then to 250 μg/ml PE. In the presence of pyruvate, some cleavage occurred at 200 μg/ml PE, with some additional cleavage fragments noted at 250 μg/ml PE Fig. (**9B**).

4. DISCUSSION

HSC-2, CAL27, and SCC1483 carcinoma cells were about twice as sensitive to the cytotoxic effects of PE towards cell proliferation than were normal gingival HF-1 fibroblasts. There are no cell culture studies in the literature comparing the responses of cancer and normal, healthy cells to PE. As noted herein, the 24-hr midpoint cytotoxicity value of PE towards the CAL27 cells was at approximately 100 μg/ml and in a 48-hr proliferation assay, Seeram *et al.* [13] noted midpoint cytotoxicity values towards CAL27 cells of approximately 50 μg/ml for punicalagin, ellagic acid, and a pomegranate tannin extract. As demonstrated herein, PE generated H_2O_2 in cell culture medium and a portion of the cytotoxic potency of PE, apparently, was due to its prooxidant nature and subsequent ability to induce oxidative stress. Cancerous cells, as compared to normal healthy cells, are known to have compromised antioxidant defense mechanisms to counteract the deleterious effects of oxidants [30]. The greater sensitivity of cancer, than of normal, cells to the prooxidant nature of EGCG [20, 31] and of extracts from natural products, including green tea [27], black tea [21], *G. biloba* [23], and apple [32], has been documented.

The prooxidant nature of nutraceutical extracts has been associated with their polyphenol constituents. For example, the polyphenols, (-)-epigallocatechin-3-gallate (EGCG) found in green tea [33], caffeic acid in coffee [34], resveratrol in red wine [35], and theaflavin-3,3'-digallate in black tea [21], exhibited prooxidant activities in cell culture media. The PE powder used herein contained a high concentration of polyphenols, i.e., >75% gallic acid equivalents, consisting of approximately 90% pomegranate hydrolysable ellagitannins [8, 36]. Apparently, the prooxidant activity of the PE was a reflection of its polyphenol content.

The extent of the generation of H_2O_2 in cell culture media amended with PE was dependent on the specific medium, with the

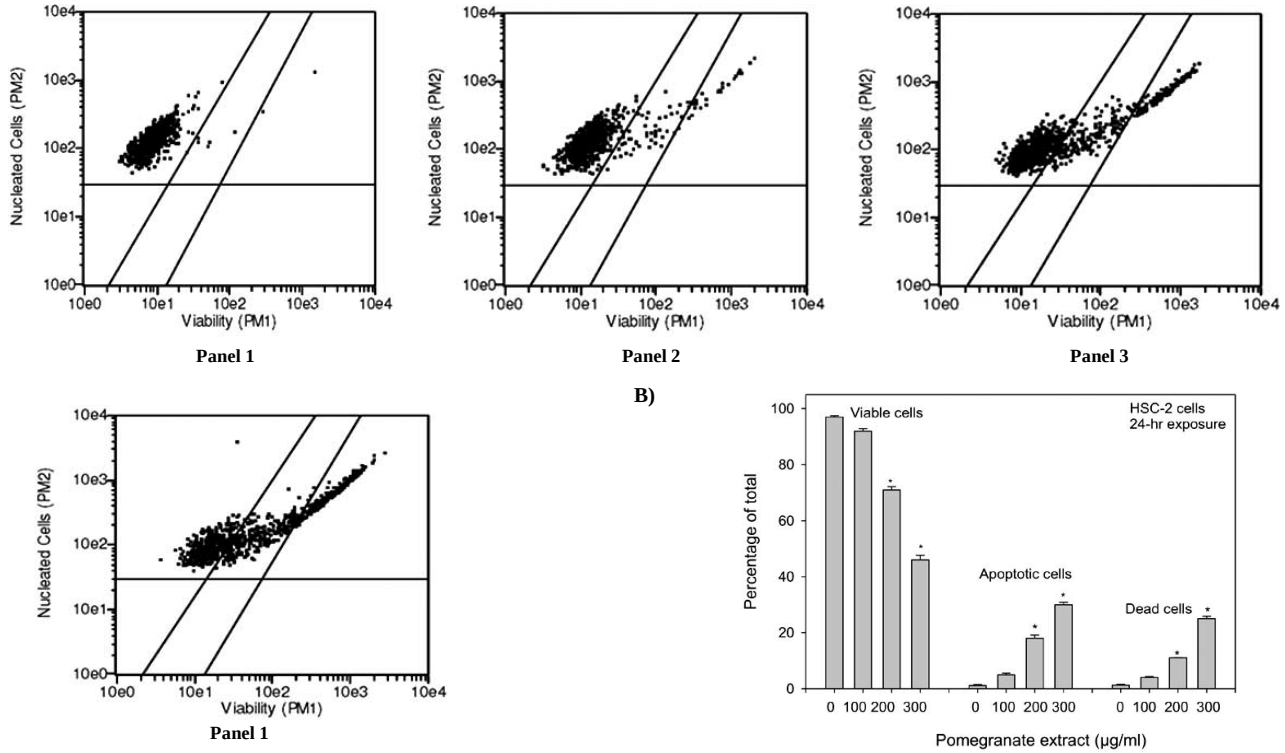

Fig. (8) (A) Representative FACS profiles of the viability and apoptosis of HSC-2 cells after a 20-hr treatment with varying concentrations of pomegranate extract. Viable cells are located on the left side of each panel; apoptotic cells between the two lines; and dead cells on the right side of the panel. Panel 1 is untreated cells, panel 2 is cells treated with 100 μg/ml extract; panel 3 is cells treated with 200 μg/ml extract, and panel 4 is cells treated with 300 μg/ml extract. **(B)** Cytometric analysis, using Guava ViaCount Reagent, of a 24-hr exposure of HSC-2 cells to increasing concentrations of pomegranate extract. Percentage of viable, apoptotic, and dead cells were quantified by the flow cytometer. The data are expressed as the arithmetic mean percent cells as viable, apoptotic, and dead ± S.E.M; * $P \leq 0.01$ as compared to untreated cells; n = 3.

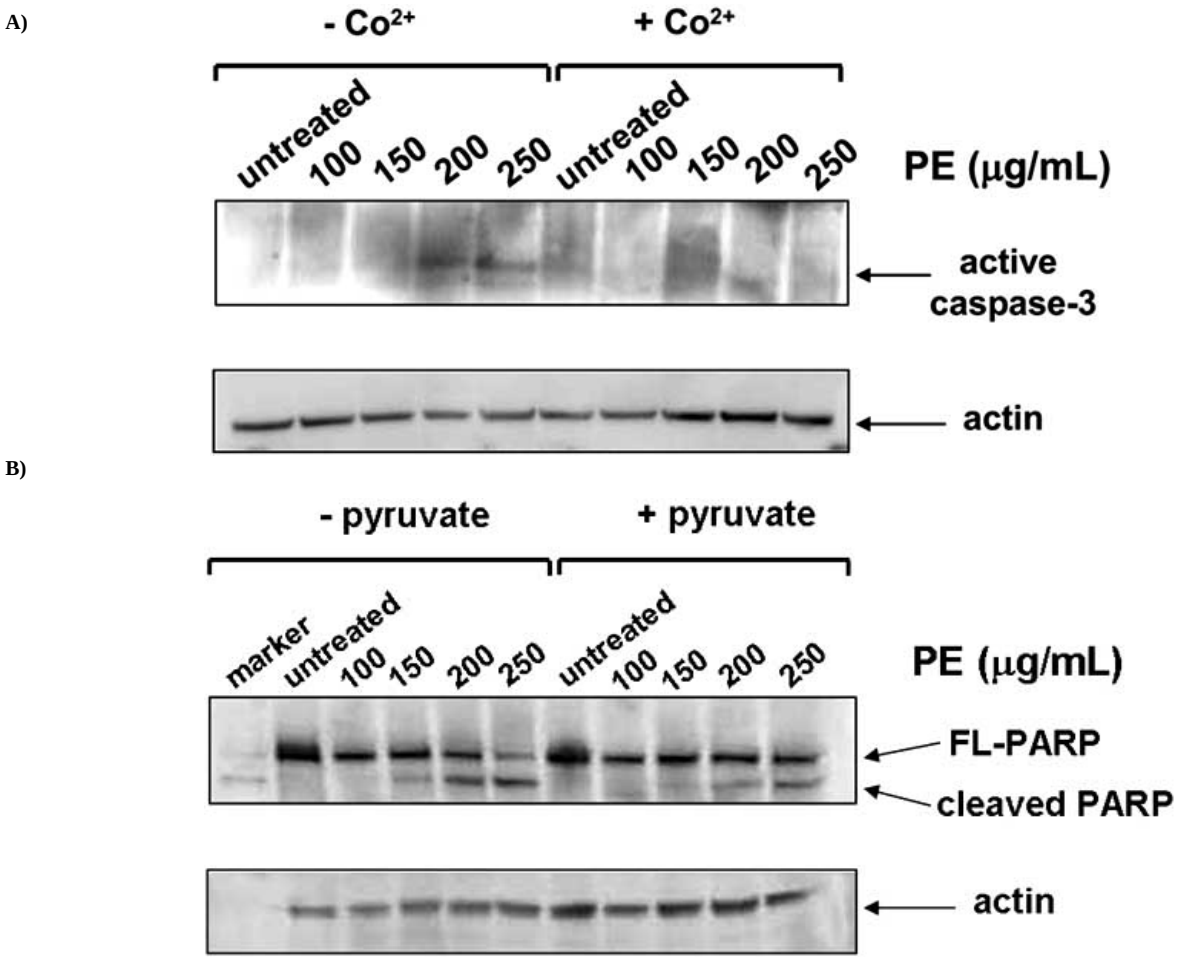

Fig. (9A). Immunoblot analysis of caspase-3 activation in HSC-2 cells untreated and after a 20-hr exposure to increasing concentrations of pomegranate extract in Dulbecco's modified Eagle medium without and with $CoCl_2$ (0.25 mM). Cellular proteins, separated by 4-20% SDS-PAGE, were transferred to nitrocellulose and probed with an antibody specific for caspase-3, which was identified by comparison to a standard molecular weight marker. An anti-actin antibody was the loading control. **(9B).** Immunoblot analysis of PARP cleavage in HSC-2 cells untreated and treated with increasing concentrations of pomegranate extract after a 20 hr exposure in Dulbecco's modified Eagle medium without and with pyruvate (110 mg/L). Cellular proteins, separated by 10% SDS-PAGE, were transferred to nitrocellulose and probed with an antibody specific for full length (FL) PARP and for the ~85 kD C-terminal fragment of PARP, which were identified by comparison to a standard molecular weight marker. An anti-actin antibody was the loading control; n = 3.

greatest amount of H_2O_2 noted in DMEM (without pyruvate). The level of H_2O_2 in PE-amended DMEM (without pyruvate) was higher than in PE-amended MEM (albeit, not statistically significant), RPMI, and McCoy's 5A media, all lacking pyruvate. Long *et al.* [37], comparing the generation of H_2O_2 in DMEM, RPMI, and McCoy's media amended with various polyphenols, including EGCG, epigallocatechin (EGC), gallic acid, and quercetin, noted that the highest levels of H_2O_2 were generated in DMEM. Essentially no H_2O_2 was detected in commercially-available DMEM with pyruvate and amended with PE. Prior studies showed the inability to detect authentic H_2O_2 added to DMEM with pyruvate [29]. Pyruvate participates in a direct oxidative decarboxylation reaction with H_2O_2 to produce acetate, CO_2, and H_2O [38].

The lesser levels of H_2O_2 detected in PE-amended exposure medium (DMEM + 2% FBS + 10% Serum Plus), as compared to PE-amended DMEM alone, reflected scavenging of the generated H_2O_2 by proteins. Depletion of H_2O_2 in PE-amended DMEM supplemented with N-acetyl-L-cysteine, and of GSH in PE-amended phosphate buffer further indicated the prooxidant nature of PE and its interactions with antioxidants.

The generation of H_2O_2 in PE-amended PBS was pH dependent, with little oxidant detected at acidic levels. An increase in the generation of H_2O_2 in PE-amended PBS as the pH was increased from 5.4 to 6.4 and then to 7.4 followed a pattern noted in PBS amended with theaflavin-3-gallate, theaflavin-3'-gallate [39], EGCG, and gallic acid [40]. Regarding EGCG, the latter researchers postulated that under alkaline conditions, the flavonoid unit of EGCG produced superoxide, which instantly yielded H_2O_2 by a dismutation reaction. Hong *et al.* [41], also in reference to EGCG, suggested that catechins are unstable at neutral and alkaline pH, undergoing oxidative polymerization with concomitant production of H_2O_2 and other reactive oxygen species. A similar mode of action may be applicable to the polyphenols in the PE.

Generation of H_2O_2 by PE implied that the induction of oxidative stress may account, at least in part, for the cytotoxic action of this phytochemical extract. The rapid depletion of authentic GSH in the presence of increasing levels of PE indicated a direct oxidation-reduction reaction and suggested that a similar mode of interaction could occur in a cellular system. Reduced GSH is an ubiquitous thiol-containing tripeptide with the key roles of maintaining intracellular redox balance and of eliminating the toxicity due to ROS. Depletion of intracellular GSH below a critical level foreshadows cellular sensitivity to oxidative stress, which then may trigger apoptosis. The intracellular level of GSH progressively decreased in HSC-2 cells treated with increasing levels of PE; this reduction of GSH in PE-treated HSC-2 cells was confirmed by fluorescent stain-

ing of intracellular thiols using Cell-Tracker™ Green CMFDA. Similarly, the prooxidant activities of EGCG [20, 42], theaflavin monogallates [39], and *G. biloba* extract [29, 43] accounted for similar depletions of intracellular GSH pools upon exposure to these test agents.

Glutathione *S*-transferase catalyses the conjugation of reduced GSH, via its sulfhydryl group, to electrophilic centers on a wide variety of substrates. CDNB, an electrophilic GSH *S*-transferase substrate, causes a rapid, irreversible depletion of GSH by way of conjugate formation, thereby making the cell susceptible to attack by ROS [44]. The sensitivity of HSC-2 cells to PE was greatly enhanced upon their prior exposure to CDNB, thus indicating the involvement of oxidative stress, in part, in the overall cytotoxicity of PE. A similar approach, using CDNB and other GSH scavengers, confirmed the induction of oxidative stress in cells exposed to EGCG [20, 45], theaflavin monogallates [39], and *G. biloba* extract [23].

That the generation of H_2O_2 by PE was sufficient to evoke a cytotoxic response was seen in the studies in which the HSC-2 cells were exposed to PE in the presence of the H_2O_2 scavengers, pyruvate, catalase, and Co^{2+}. Catalase enzymatically and Co^{2+} catalytically [46] decomposes H_2O_2 to H_2O and O_2. H_2O_2 through an oxidative decarboxylation reaction converts pyruvate to acetate, H_2O and CO_2 [38]. The cytotoxicity of PE to the HSC-2 cells was lessened, but not completely eliminated, upon coexposures with these H_2O_2 scavengers, suggesting that other mechanisms, aside from the induction of oxidative stress were involved in the overall cytotoxic effect. Scavenging of H_2O_2 by Co^{2+}, catalase [47], and pyruvate [23] also protected cells against oxidative injury by EGCG.

H_2O_2 and ROS, in general, induce apoptosis, which may be prevented by exogenous catalase, GSH, and *N*-acetylcysteine or by elevated intracellular levels of GSH [48]. Although the anticarcinogenic activities of nutraceuticals have been associated with their antioxidant activities, there is a growing body of literature, primarily from studies with cell culture, that natural plant extracts have sufficient prooxidant activity to inhibit cell proliferation and, thereafter, to trigger apoptosis, particularly in cancer cells [17, 25, 49-51]. Exposure of the HSC-2 cells to PE induced apoptosis, as indicated by flow cytometry and by caspase-mediated cleavage of PARP, an intracellular marker of apoptosis. Specific polyphenol constituents in pomegranate juice, including ellagic acid [5, 52, 53] and punicalagin [13, 54], and various pomegranate extracts [5, 13, 55] induced apoptosis in a variety of human cancer cells. These latter studies with pomegranate extracts either noted only that apoptosis was induced [13, 55] or noted that the induction of apoptosis correlated with effects on cell signaling. For example, studies with human prostate cancer cells showed treatment with pomegranate extract increased JNK phosphorylation and decreased Akt and mTOR activation, events that were consistent with a growth inhibitory and proapoptotic function [5]. Studies by Malik *et al.* [56] showed that the induction of apoptosis of prostate cancer cells treated with pomegranate extract was associated with upregulation of proapototic Bax and Bak, downregulation of antiapoptotic Bcl-X_L and Bcl-2, and induction of WAF1/p21 and KIP1/p27, known to regulate entry of cells at the G_1-S phase transition checkpoint and to induce apoptosis. In these studies, the involvement of oxidative stress was not evaluated. In the presented studies herein, the induction of PE-induced apoptosis (as shown by activation of caspase-3 and by PARP fragmentation) was lessened in the presence of exogenous scavengers of H_2O_2 (i.e., cobalt and pyruvate, respectively), thus, indicating that the prooxidant nature of the extract was a factor in the induction of apoptosis.

Halder *et al.* [3] and Li *et al.* [4] noted that consumption of teas could mitigate oral precancerous lesions. Lambert *et al.* [57] detected the production of salivary H_2O_2 in individuals chewing green tea leaves or holding a green tea solution in the oral cavity. Lambert and Elias [58] postulated that the generation of sufficient levels of ROS by elevated amounts of tea polyphenols may be important in clearing transformed cells from the body and in limiting tumor growth. Conversely, lesser levels of polyphenols may evoke low level oxidative stress, leading to activation of the antioxidant response element (ARE)/Nft-2 system, promoting the synthesis of endogenous antioxidants (e.g., enzymes to synthesize GSH). This increased endogenous antioxidant capacity may be of more importance prior to carcinogen exposure.

The polyphenolic content of PE is higher than that in teas [59]. The findings presented herein indicate that pomegranate extract, as previously suggested with green tea polyphenols [2, 58, 60], may have the potential as a chemopreventive agent for the oral cavity.

CONFLICT OF INTEREST

None

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