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Cytoprotective effect of preparations from various parts of *Punica granatum* L. fruits in oxidatively injured mammalian cells in comparison with their antioxidant capacity in cell free systems

Piero Sestili [a,b,*], Chiara Martinelli [b], Donata Ricci [c], Daniele Fraternale [c], Anahi Bucchini [c], Laura Giamperi [c], Rosanna Curcio [c], Giovanni Piccoli [b,d], Vilberto Stocchi [b,d]

[a] *Istituto di Farmacologia e Farmacognosia, Università degli Studi di Urbino "Carlo Bo", Via S. Chiara, 27-61029 Urbino, Italy*
[b] *Istituto di Ricerca sull'Attività Motoria, Via i Maggetti 26, Loc. Sasso, Università degli Studi di Urbino "Carlo Bo", Urbino, Italy*
[c] *Istituto di Botanica e Orto Botanico "P. Scaramella", Università degli Studi di Urbino "Carlo Bo", Urbino, Italy*
[d] *Istituto di Chimica Biologica "G. Fornaini", Università degli Studi di Urbino "Carlo Bo", Urbino, Italy*

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Abstract

Pomegranate (*Punica granatum* L.) juice (PJ) is being increasingly proposed as a nutritional supplement to prevent atherosclerosis in humans. This therapeutically valuable potential has been attributed to PJ antioxidant capacity which has been mostly tested by means of cell-free assays: indeed, to the best of our knowledge, no study has focused on the direct antioxidant capacity of PJ in cultured cells. Here, the antioxidant capacity in cell free-systems of preparations from various parts of pomegranate has been compared with their cytoprotective – *bona fide* antioxidant – activity in cultured human cells (U937 promonocytes and HUVEC endothelial cells) exposed to an array of oxidizing agents. Pomegranate derivatives were PJ, arils only juice (AJ) and aqueous rinds extract (RE). In cell-free assays – 1,1-diphenyl-2-picrylhydrazyl (DPPH), chemiluminescence luminol/xanthine/xanthine oxidase and lipoxygenase assays – all the preparations displayed good antioxidant capacity, the relative potency order being RE > PJ > AJ. On the contrary, only RE was capable of preventing the deleterious effects – cytotoxicity, DNA damage and depletion of non-protein sulphydrils (NPSH) pool – caused by treatment of cells with H_2O_2, *tert*-butylhydroperoxide (tB-OOH) or oxidized lipoproteins (Ox-LDL) via a mechanism which is likely to involve both direct scavenging of radical species and iron chelation. Surprisingly, AJ and PJ slightly sensitized cells to the cytotoxic effects of the three agents. Then it would appear that AJ, the major and tasty part of PJ, does not contain ellagic acid and punicalagin (i.e. the polyphenols highly represented in RE which are reputed to be responsible for the antioxidant capacity) in amounts sufficient to exert cytoprotection in oxidatively injured, living cells. Based on these results, the development and evaluation of rinds-only based derivatives for antiatherogenic preventive purposes in humans should be encouraged.

Keywords: Pomegranate; Antioxidants; Oxidative stress; Cytotoxicity; Atherosclerosis; Chemoprevention

1. Introduction

The notion that oxidative stress exerts a concausal role in a wide number of chronic and degenerative pathologies has led to a considerable effort in search of antioxidant compounds to prevent the onset and counteract the progression of these maladies [1–3]. Indeed, in the past two decades particular interest has been devoted to the identification of naturally occurring antioxidants from edible plants and fruits [3–5]. Fruits and vegetables contain different antioxidant compounds, such as vitamin C, vitamin E and carotenoids, whose activities have been established in recent years, but these compounds are not the only ones contributing to their antioxidant capacity [6]. In particular, the presence of polyphenol compounds, such as flavonoids also contributes to the beneficial effect of this group of foods [7–10], interacting with various biological systems [11].

Abbreviations: AJ, arils juice; DPPH, 1,1-diphenyl-2-picrylhydrazyl; HUVEC, human umbilical vein endothelial cells; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide; NDF, nuclear diffusion factor; Ox-LDL, oxidized lipoproteins; PDER, pomegranate derivatives; PJ, pomegranate juice; RE, rinds extract; tB-OOH, *tert*-butylhydroperoxide

* Corresponding author at: Istituto di Farmacologia e Farmacognosia, Università degli Studi di Urbino "Carlo Bo", Via S. Chiara, 27-61029 Urbino, Italy. Tel.: +39 0722 303414; fax: +39 0722 303401.
E-mail address: sestili@uniurb.it (P. Sestili).

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There is a wealth of anecdotal and mythological reports regarding the pomegranate (*Punica granatum* L.) fruit. Ancient Mediterranean civilizations, as well as Persians and Babylonians, attributed supernatural properties to pomegranate fruits and seeds [12]. The fruits have also been long and widely used in folk and traditional medicine for the treatment of a number of pathologies [12,13].

Pomegranate belongs to the family of Punicaceae, is grown mainly in the Near East India, Spain (southeastern), Israel and the United States (California). A significant economic importance is given to the fruits which are either consumed fresh or used commercially in the juice, jam and wine industries [14].

The edible portion of pomegranate fruit (50% of total fruit weight), called arils, originates from the pith (anchoring tissue) and is well protected by carpellary membranes (rinds) [12]. Arils are rich in sugars, vitamins, polysaccharides, polyphenols and minerals, and seeds are rich in polyunsaturated (n-3) fatty acids [15]. The three major anthocyanidins (polyphenols) present in arils are delphinidin, cyanidin and pelargonidin [16]. Punicalagin (an ellagic tannin) is the major chemical constituent of pith and rinds [17], and is a natural potent antioxidant present in fruit waste. The most popular and commercially valuable pomegranate derivative (PDER) is pomegranate juice (PJ) which contains 85% water, 10% total sugars, 1.5% pectin, ascorbic acid, and polyphenolic flavonoids [12]. In PJ, fructose and glucose are present in similar quantities; the soluble polyphenol content varies within the limits of 0.2–1.0%, depending on the variety, and includes anthocyanins, catechins, ellagic tannins, and gallic and ellagic acids [12,18]. Due to its polyphenols, tannins and anthocyanins content PJ possesses antioxidant capacity which has been extensively documented in cell-free systems [11,13–15,17,19–23]. The antioxidant capacity is thought to contribute to the pharmacologically valuable properties which have been reported: recent studies indicate that PJ elicits antiatherogenic and vasoprotective effects in mice and humans [12,24–30], exerts beneficial effects on oxidation-sensitive genes at sites of perturbed shear stress [29] and can inhibit cyclooxygenases and lipoxygenases [28]. Industrial PJ properties seem to depend on the fruit cultivar and processing methods [14,31], which do not appear to be fully standardized [14,31]. Some studies [14,17,20,22] reported that parts of pomegranate usually considered as by-products (which include the whole pomegranate fruit left after juice preparation such as rind membranes) display significant antioxidant capacity: interestingly, the specific activity of these parts, as tested in cell-free systems [14,17,20], seems higher than that of arils and PJ, and an indication in this direction has been very recently reported in an in vivo study [30]. However, most of the research work comparing the antioxidant capacity of various parts from pomegranate fruit has been carried out exclusively in cell-free systems and, to the best of our knowledge, no research has been specifically aimed to directly and systematically test the antioxidant potential of these parts on oxidatively injured cultured cells. The importance of testing antioxidant capacity in cell cultures is that the direct antioxidant capacity of plant derivatives in cell-free systems may not necessarily correlate with that exhibited in more complex biological systems, including cultured cells. In these systems, the actual antioxidant capacity of the compounds may be confounded by other biological activities, or simply by the cellular pharmacology of each component (metabolism, uptake, egress). The lack of data regarding this issue, along with the recent finding that rinds seem to possess a remarkable antioxidant activity, prompted this study. We report the determination of the antioxidant capacity of experimental, microfiltered whole fruit PJ, arils-only juice (AJ) or rinds-only extract (RE) in cultured human promonocytes and endothelial cells exposed to an array of oxidative agents and compare these results with those from cell-free systems.

2. Materials and methods

2.1. Reagents

Reagent-grade chemicals, *tert*-butylhydroperoxide (tB-OOH), H_2O_2, 1,1-diphenyl-2-picryl-hydrazil (DPPH), luminol, xanthine, xanthine oxidase, linoleic acid, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT), Elman's reagent and trolox were from Sigma–Aldrich (Milan, Italy). Cell culture media, sera and antibiotics were from Cambrex Bio Science Srl (Milan, Italy). Oxidized lipoproteins (Ox-LDL) were a generous gift of Prof. Alberico Catapano (Department of Pharmacological Sciences, University of Milan, Italy) and were prepared as detailed in Ref. [32]. Ox-LDL were used for experiments within 5 days from their preparation.

2.2. Preparation of PJ, AJ and RE

Punica granatum fruits were collected in Urbino (458 m above sea level) in September 2005 and identified by D. Fraternale. A voucher specimen is deposited in the herbarium of the Botanical Garden of the University of Urbino, Italy. PJ was prepared as follows: the whole fruit was cut in half and squeezed in an electric lemon-squeezer and the resulting juice was then centrifuged at 2500 rpm for 10 min. AJ was obtained from arils manually separated, crashed in a mortar and centrifuged at 2500 rpm for 10 min. RE was prepared from rinds which were manually removed, crashed in a mortar with Tris/HCl 50 mM pH 7,5 (1:2, w/v) and centrifuged at 2500 rpm for 10 min. All these steps were performed at ice bath temperature. The supernatants from the centrifugation step of the three PDERs were recovered, microfiltered, aliquoted and immediately stored at −20 °C. The dry weights of PJ, AJ and RE were 0.459, 0.61 and 0.39 g ml^{-1}, respectively. Total polyphenol content was determined by the Prussian Blue method [33].

2.3. Determination of the antioxidant capacity in cell-free systems

The antioxidant capacity of PJ, AJ and RE was evaluated using three different methods: DPPH assay [34], chemiluminescence luminol/xanthine/xanthine oxidase test [35] and 5-lipoxygenase inhibition assay [35]. The DPPH assay was conducted as follows: a 100 μM DPPH ethanolic solution was prepared and 1.5 ml was added to the same volume of sam-

ple diluted in 50 mM Tris/HCL. The absorbance decrease at 517 nm was recorded after 10 min and the percent decrease (corrected for the control, without antioxidant agents added) was taken as an index of the antioxidant capacity. Chemiluminescence luminol/xanthine/xanthine oxidase assay has been carried out as described by Sud'ina et al. [35], with minor modifications. Reaction buffers consisted in 1 ml of 20 mM Na_2CO_3/$NaHCO_3$ pH 10.0, 0.1 M EDTA, 1 mM NaN_3 and 50 μM xanthine, 1 μM luminol and the PPR sample (or the same volume of distilled water Tris/HCL as a blank). The reaction was started by adding 0.02 μg ml^{-1} commercial xanthine oxidase and readings have been made at 30 s intervals for 15 min at room temperature in a liquid scintillation counter. For lipoxygenase assay, 5-lipoxygenase (0.18 μg ml^{-1}) was added to the reaction mixture (1 ml pre-equilibrated at 20 °C for 20 min containing 10 mM linoleic acid, the sample or the same quantity of Tris/HCL as reference, and 50 mM sodium phosphate, pH 6.8) and the formation of hydroperoxides from linoleic acid was then observed spectrophotometrically at 235 nm at 20 °C.

2.4. Spectrophotometric determination of iron chelation

The ability of RE to chelate iron was determined spectrophotometrically [5]. UV–vis absorption spectra (200–550 nm) were measured using a Beckman DU640B spectrophotometer (Beckman Coulter Inc, Fullerton, CA, USA). RE was diluted, and *o*-phenanthroline was dissolved, in phosphate buffered saline (50 mM, pH 7.4) and the spectral changes were measured in the absence or presence of 50 μM $FeSO_4$.

2.5. Cell culture and treatments

Cells were grown at 37 °C in an atmosphere of 95% air and 5% CO_2. Human promonocytic U937 cells were cultured in suspension in RPMI 1640 medium supplemented with antibiotics (100 U ml^{-1} penicillin, 100 μg ml^{-1} streptomycin), 1.2 mM glutamine and 10% fetal bovine serum. For experiments, cells were resuspended at a number of 4×10^5 cells/treatment condition. Human umbilical vein endothelial cells (HUVEC) were maintained in M199 medium containing antibiotics (100 U ml^{-1} penicillin, 100 μg ml^{-1} streptomycin), 1.4 mM glutamine, 10% fetal bovine serum and 50 μg ml^{-1} endothelial cell growth factor. At the treatment stage the cell number was kept between 4 and 5×10^5/35 mm well. Stock solutions of H_2O_2 and tB-OOH were freshly prepared in distilled water and diluted to the proper concentrations. For experiments, cells were washed and the growth medium replaced with 2 ml of saline A (8.182 g l^{-1} NaCl, 0.372 g l^{-1} KCl, 0.336 g l^{-1} $NaHCO_3$ and 0.9 g l^{-1} glucose) prewarmed at 37 °C and PJ, AJ or RE were added to cultures and equilibrated for 20 min before addition of the oxidants. For cytotoxicity, cells were treated with the oxidants for 60 min in saline A (H_2O_2 or tB-OOH) or for 6 h in complete culture medium (Ox-LDL). Finally, for DNA damage experiments the treatments were similar to those detailed for cytotoxicity, with the exception that cells were exposed to tB-OOH for 30 min.

2.6. Cytotoxicity assays

The cytotoxic response was evaluated after 48 h post-challenge growth in complete culture medium, this time interval allows to quantify the extent of growth arrest and cell death caused by the oxidants in treated cultures versus logarithmically growing control cells. Two independent and unrelated techniques were adopted, namely trypan blue exclusion assay and MTT assay.

Trypan blue exclusion assay: an aliquot of cell suspension was diluted 1:1 with 0.4% trypan blue and the cells were counted with a haemocytometer. Results are expressed as "percent survival", that is the percent ratio of viable (unstained) cells in treated versus untreated samples.

MTT assay: MTT was added (50 μg ml^{-1}) to each sample (cell suspensions for U937 or monolayers for HUVEC cells). The cells were then incubated for a further 1 h at 37 °C, washed and dissolved in 2 ml of dimethyl sulfoxide. Formation of blue formazan was measured spectrophotometrically at 570 nm.

2.7. Determination of non-protein sulphydrils (NPSH)

Cellular NPSH levels were quantified spectrophotometrically in metaphosphoric acid extracts with the Elman's reagent as described in [36].

2.8. Fast halo assay

The assay has been carried out as described in [37]. Briefly, immediately after the treatments, the cells were resuspended at 4.0×10^4 /100 μl in ice-cold phosphate-buffered saline (8 g l^{-1} NaCl, 1.15 g l^{-1} Na_2HPO_4, 0.2 g l^{-1} KH_2PO_4, 0.2 g l^{-1} KCl) containing 5 mM EDTA: this cell suspension was diluted with an equal volume of 2.0% low-melting agarose in phosphate-buffered saline and immediately sandwiched between an agarose-coated slide and a coverslip. After complete gelling on ice, the coverslips were removed and the slides were immersed in NaOH 0.3 M for 15 min at room temperature. Ethidium bromide (10 μg ml^{-1}) was directly added to NaOH during the last 5 min of incubation. The slides were then washed and destained for 5 min in distilled water. The ethidium bromide-labelled DNA was visualized using a Leica DMLB/DFC300F fluorescence microscope (Leica Microsystems, Wetzlar, Germany) and the resulting images were digitally recorded on a personal computer and processed with an image analysis software (Scion Image). The extent of strand scission has been quantified by calculating the nuclear diffusion factor (NDF), which represents the ratio between the total area of the halo plus nucleus and that of the nucleus.

3. Results

The results obtained showed that the preparations from various parts of *Punica granatum* fruits possess a significant antioxidant capacity as determined with three different acellular, in vitro tests, namely DPPH assay, 5-lipoxygenase assay and

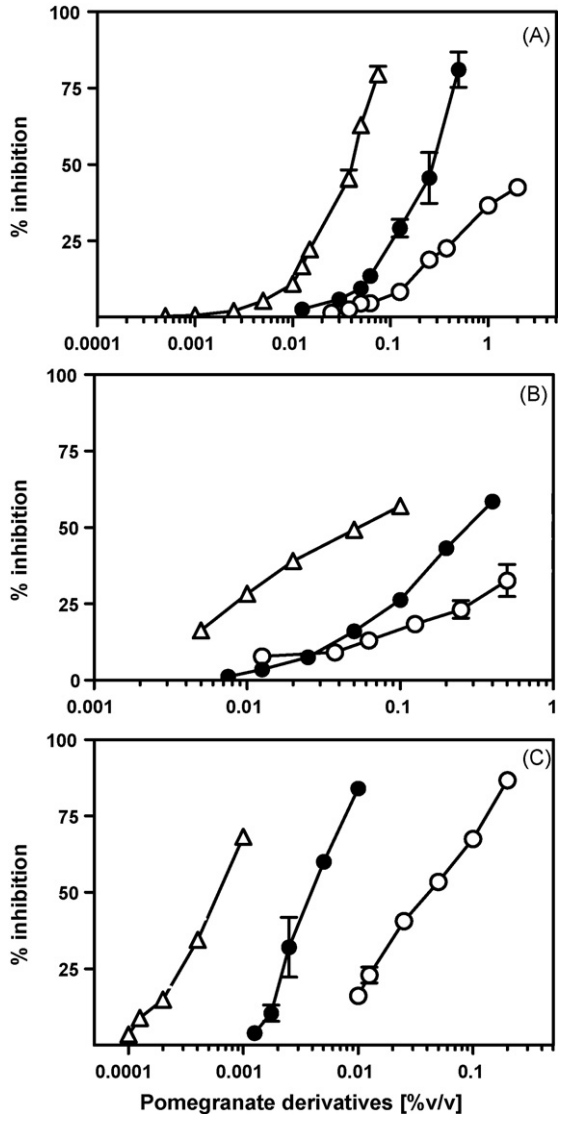

Fig. 1. Antioxidant capacity of PJ, AJ and RE in the DPPH test (A), lipoxygenase assay (B) and luminol/xanthine/xanthine oxidase chemiluminescence assay (C). The antioxidant capacity was assayed as detailed in Section 2 in the presence of increasing concentrations of PJ (closed circles), RE (triangles) or AJ (open circles). Results represent the percentage of inhibition induced by increasing concentrations (expressed as %, v/v in the sample buffers) of PJ, AJ or RE on the production of DPPH radicals, hydroperoxide formation and chemiluminescence emission, and are the means S.E.M. from 4 to 5 separate determinations.

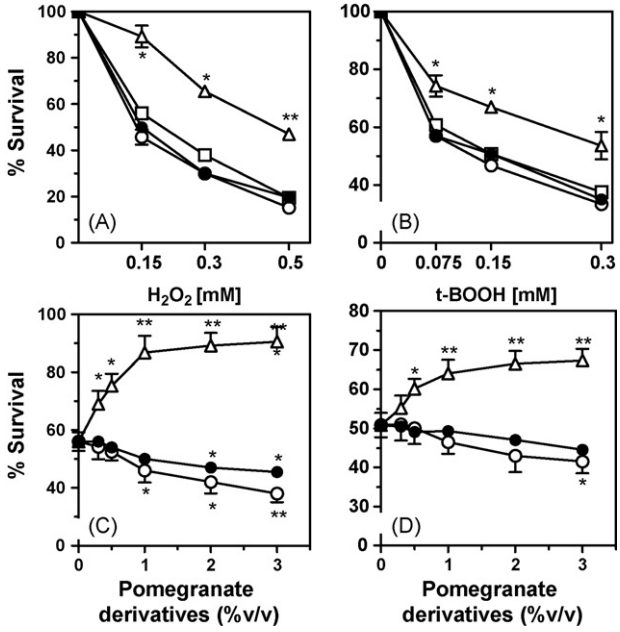

Fig. 2. Effect of PJ, AJ and RE on H_2O_2 or tB-OOH-induced cytotoxicity in U937 cells as a function of either PDER- or oxidant-concentration. Panels A and B: cells were pre-incubated in Saline A for 20 min with 0 (squares) or 1%, v/v of PJ (closed circles), RE (triangles) or AJ (open circles); at this time increasing concentrations of H_2O_2 (A) or tB-OOH (B) were added and, after 60 min, cells were washed and re-seeded in complete culture medium. Cell survival was determined after 48 h of growth using the trypan blue exclusion assay. Panels C and D: cells were subjected to the same experimental procedures described in (A) and (B), with the only exception that cultures were exposed to a fixed dose (0.15 mM) of H_2O_2 (C) or tB-OOH (D) with or without increasing concentrations of PJ (closed circles), RE (triangles) or AJ (open circles). Results represent the means ± S.E.M. from 10 separate experiments. $^{*}P < .05$ and $^{**}P < .005$ (unpaired *t*-test) compared to PDERs-unsupplemented cells.

luminol/xanthine/xanthine oxidase chemiluminescence assay (Fig. 1A, B and C, respectively). These data are in agreement with previously published observations [20]. The different polyphenol content of the various parts of the fruit correlates directly with the different antioxidant capacity of their corresponding derivatives, i.e. the higher the amount of polyphenols the higher the antioxidant capacity. Indeed, the highest polyphenol content was detected in RE (7.48 mg polyphenol ml^{-1} of extract), followed by PJ (1.54 mg polyphenol ml^{-1} of juice) and AJ (0.57 mg polyphenol ml^{-1} of juice), and the same order of antioxidant capacity could be observed with the three assays (Fig. 1A–C). In particular, RE proved to be 1 to 3 orders of magnitude more active than PJ and AJ: at this regard, however, other non-quantitative factors possibly concurring to determine RE high antioxidant capacity, such as its phenolic composition, cannot be excluded.

The second set of experiments was aimed to assess the antioxidant capacity of PJ, AJ and RE in oxidatively injured cultured cells. For this purpose the effect of these preparations on the cytotoxic response of U937 promonocytic cells exposed to H_2O_2 (Fig. 2A) or tB-OOH (Fig. 2B) was investigated. PJ, AJ and RE were the same utilized in cell-free determinations, and the concentrations selected for this set of experiments (up to 3%, v/v) were not cytotoxic *per se* (not shown). 1% (v/v) concentrations of RE, PJ and AJ corresponded to a final polyphenol content of 74.8, 15.4 and 5.7 μg ml^{-1}, respectively. Under the selected treatment conditions (see Section 2), exposure to either H_2O_2 or tB-OOH resulted in a dose-related reduction of surviving cells (Fig. 2A and B) and, according to previous studies [38–40], the mode of cell death we observed was necrosis.

Contrary to expectations, the three PDERs did not exert univocal protective effects on oxidatively injured U937 cells: in particular, upon H_2O_2 exposure, only RE was cytoprotective while, surprisingly, AJ and, to a lesser extent PJ, sensitized cells to oxidative attack (Fig. 2A). Similar results were obtained in tB-OOH-treated cells where RE, again, was cytoprotective (although to a lesser extent as compared to H_2O_2 treated-cells),

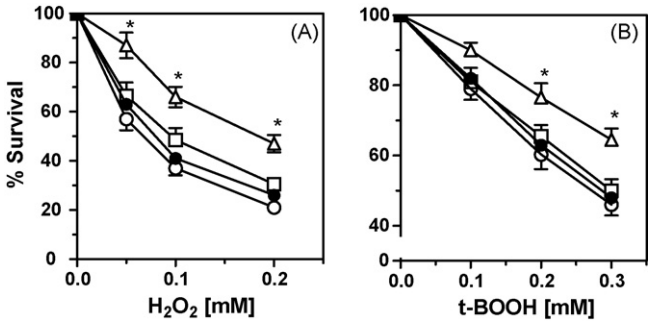

Fig. 3. Effect of PJ, AJ and RE on oxidant-induced cytotoxicity in HUVEC cells. Cells were pre-incubated in Saline A for 20 min with 0 (open squares) or 1%, v/v of PJ (closed circles), RE (triangles) or AJ (open circles); at this time increasing concentrations of H_2O_2 (A) or tB-OOH (B) were added and, after 60 min, cells were washed and grown in drug-free, complete medium. Cell survival was determined after 48 h of growth using the trypan blue exclusion assay. Results represent the means ± S.E.M. from seven separate experiments. $^{*}P < .05$ and $^{**}P < .005$ (unpaired *t*-test) compared to PDERs-unsupplemented cells.

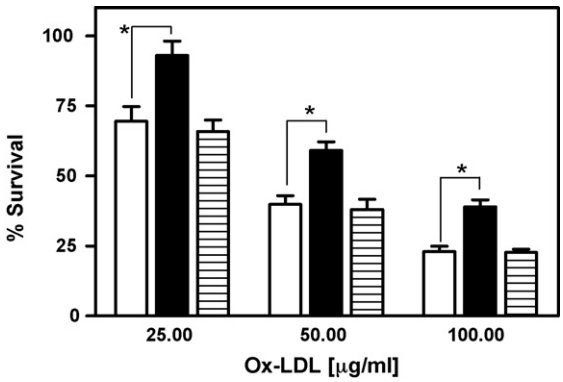

Fig. 4. Effect of PJ, AJ and RE on Ox-LDL-induced cytotoxicity in HUVEC cells. Cells were treated for 6 h in serum-free medium with increasing concentrations of Ox-LDL in the absence (open bars) or presence of 1% (v/v) RE (solid bars) or AJ (striped bars); cells were then washed and grown in drug-free, complete medium. Cell survival was determined after 48 h of growth using the trypan blue exclusion assay. Results represent the means ± S.E.M. from three separate experiments, each performed in duplicate. $^{*}P < .01$ (unpaired *t*-test) compared to PDERs-unsupplemented cells.

and AJ or PJ were not (Fig. 2B). The effect of increasing concentrations of PDERs on the cytotoxic response elicited by a fixed dose of H_2O_2 or tB-OOH in U937 cells has been tested and results are shown in Fig. 2C and D: under these conditions only RE afforded significant cytoprotection. The effect of RE was linear up to 1% and tended to a plateau at higher concentrations. AJ, and to a lesser extent PJ, sensitized U937 cells to H_2O_2 or tB-OOH insult (Fig. 2C and D). Finally, longer preincubation times (up to 3 h) with AJ or PJ did not result in any cytoprotection in H_2O_2 challenged U937 cells (not shown).

In order to investigate and compare the effects of PJ, AJ and RE in a different cell line, HUVEC cells were subjected to treatments similar to those described for U937. As expected, increasing concentrations of H_2O_2 or tB-OOH were cytotoxic to HUVEC (Fig. 3A and B) and, according to previous observations [41], promoted extensive necrotic cell death which could be mitigated only by RE; again, addition of PJ or AJ slightly enhanced oxidant-induced cytotoxicity.

Since vascular endothelium is a critical and pathologically relevant target of the deleterious activity of Ox-LDL [42,43], the effect of the three PDERs on Ox-LDL-treated HUVEC cells was also studied: results obtained (Fig. 4) are qualitatively similar to those described above, i.e. RE, unlike PJ and AJ, was cytoprotective to Ox-LDL-treated HUVEC cells. With regard to cell survival experiments, all the results described so far have been confirmed using the MTT assay, an independent, unrelated method for cytotoxicity determination which gave quantitatively similar outcomes (not shown).

The fact that AJ and PJ slightly sensitized cells to the toxic effects of the oxidants, prompted us to test whether their presence could affect the rate of cellular NPSH (of which glutathione represents 90% or more [36] and which is a sensitive cellular target of oxidative attack) consumption in oxidatively injured cells. As expected, challenge of U937 cells with 0.5 mM H_2O_2 for 1h provoked a marked drop of NPSH levels (53.3% as compared to control cells) and, interestingly, the presence of AJ significantly, and PJ slightly, reduced the level of NPSH. Conversely, addition of RE resulted in a significant NPSH sparing, which is likely to parallel the cytoprotective action (Fig. 5).

The next series of experiments focused on the activity of RE, the only derivative displaying antioxidant/cytoprotective capacity in the previous experiments. Since it is thought that the antioxidant effect of plant derived polyphenols may derive either from their scavenging activity, or from the ability to chelate intracellular iron ions [5,39], further experiments have been aimed to gain information on the relative contribution of these two mechanisms to the actual RE antioxidant capacity. Interestingly, in vitro measurement of the UV–vis absorption spectrum for RE showed, upon addition of Fe^{2+} ions, a characteristic absorbance band shift indicative of the formation of iron chelates (Fig. 6A). To see whether iron chelation might be biologically relevant and contribute to the actual cytoprotection of RE, U937 cells were exposed to tB-OOH with or without RE

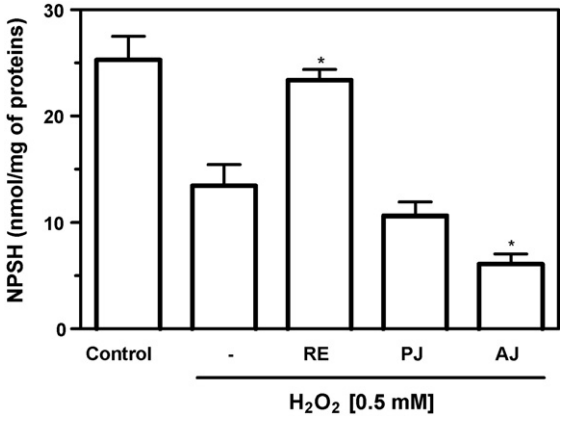

Fig. 5. Effect of AJ, PJ or RE on H_2O_2-injured U937 cells NPSH levels. Cells were treated as detailed in the legend to Fig. 2A with or without RE, AJ or PJ (final concentration of PDERs was 1%, v/v) and treated for 60 min with 0.5 mM H_2O_2. NPSH levels were immediately determined with the DTNB assay as detailed in Section 2. Results represent the means ± S.E.M. from four separate experiments. $^{*}P < .05$ and $P < .005$ (unpaired *t*-test) compared to H_2O_2-alone treated cells.

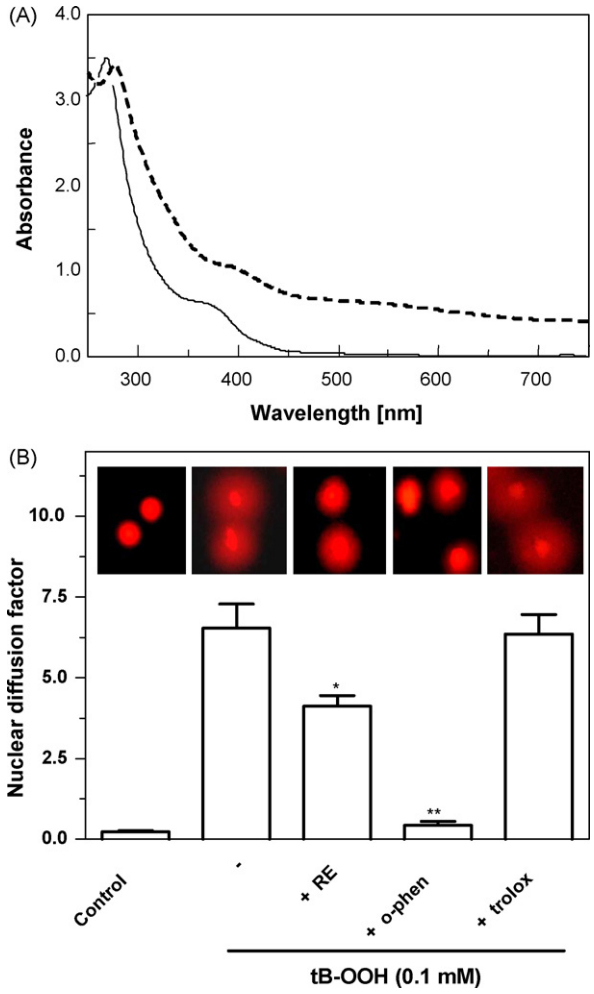

Fig. 6. RE chelates divalent iron and reduces tB-OOH-induced nuclear damage. Panel A: RE was diluted (1%, v/v) in phosphate buffer (50 mM, pH 7.4) in the absence or presence (striped line) of 50 μM $FeSO_4$ and spectra (200 to 500 nm) were immediately recorded. Panel B: U937 cells were treated in saline A without or with RE (1%, v/v), *o*-phenanthroline (10 μM) or trolox (0.1 mM) and, after 20 min, tB-OOH (0.1 mM) was added to the cultures, which were further incubated for 30 min: DNA damage was then assayed with the fast halo assay, as detailed in Section 2. The NDF value (0.23 ± 0.0031) of untreated cells is also shown. Data are the mean ± S.E.M. from three separate experiments, each performed in duplicate. $^{*}P < .05$ and $P < .005$ (unpaired *t*-test) compared to PDERs-unsupplemented cells. Representative micrographs showing the appearance of nuclei and halos in each treatment condition are placed over the corresponding bars of Panel B.

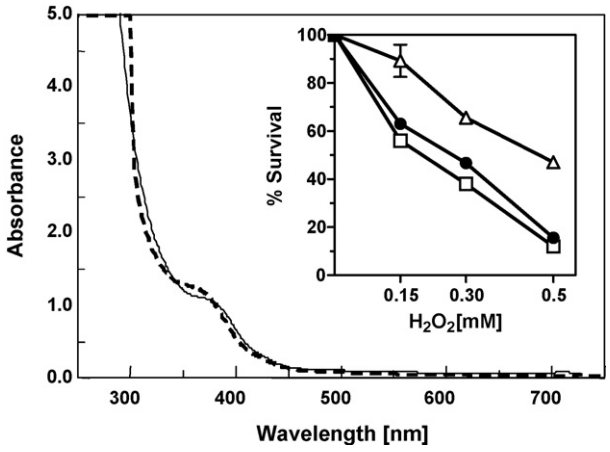

Fig. 7. Effect of room temperature storage on Fe-chelating and cytoprotective activity of RE. RE was kept at room temperature for 7 days, and the iron chelating- (main graph) and cytoprotective activity (inset) of the aged extract were then tested as detailed in Figs. 2 and 6, respectively. Final concentration of aged and control RE was 1%. Main graph: continuous line and striped line correspond to the spectra of 7 days-aged RE without or with 50 μM $FeSO_4$, respectively. Inset: survival curves for cells treated with H_2O_2 alone (squares), H_2O_2 plus fresh RE (triangles) or H_2O_2 plus 7 days-aged RE (circles) are shown.

and assayed for DNA single strand breakage. It is well known, indeed, that iron chelating-plant polyphenols, unlike those possessing exclusively radical scavenging activity, are capable of preventing tB-OOH-induced nuclear oxidative damage [5,44]. tB-OOH treated cells displayed, upon fast halo assay analysis, a large halo of single stranded DNA fragments concentric to the nuclear remnant (see the second micrograph from left in Fig. 6B), which is indicative of a high level of DNA single strand breakage [37]. Interestingly, this phenomenon could be hampered by the presence of RE, completely prevented by the reference iron chelator *o*-phenanthroline and not affected by the reference scavenger trolox (results and micrographs in Fig. 6B). Then, RE-polyphenols seem to be capable of chelating intracellular iron and this activity, in analogy with other iron-chelating polyphenols such as quercetin [5,44], is likely to contribute to their actual antioxidant capacity.

Since it has been reported that pomegranate looses its antioxidant capacity even upon long-term storage at −20 °C [14], the last set of experiments was aimed to see whether conditions mimicking shelf-storage might alter RE cytoprotective activity in H_2O_2-treated U937 cells. RE (1 ml aliquots in 15 ml plastic tubes) was then kept at room temperature for up to one week and its activity was tested daily. Results indicate that RE cytoprotective activity decreases linearly over time (not shown) and disappears whithin the first week of storage at room temperature (Fig. 7, inset). Interestingly, the UV–vis absorption spectrum for 7 days-aged RE did not show, upon addition of Fe^{2+} ions, the characteristic absorbance band shift which had been observed with −20 °C stored-RE (compare the main graph in Fig. 7 with Fig. 6A).

4. Discussion

Pomegranate fruits have been extensively used in folk medicine for a number of beneficial effects. Today, the use of pomegranate in fresh and beverage forms is on the rise due to the well known in vitro antioxidant and in vivo antiatherogenic properties. It is believed that the antiatherogenic effects derive from the antioxidant capacity of pomegranate constituents, among which the most active fraction is the polyphenolic one. Interestingly, it has also been reported that preparations (extracts or juices) from different parts (arils, rinds or whole fruit) of pomegranate display diverse antioxidant potency [14,17,20,22].

Despite the fact that a wealth of studies has described and characterized PDERs antioxidant capacity in cell free systems [11,13,14,16,17,19–22,27], no information exists on their cyto-

protective, *bona fide* antioxidant capacity in oxidatively injured cultured mammalian cells.

To the best of our knowledge, this is the first research aimed at determining the antioxidant and cytoprotective activity of different PDERs, namely PJ, RE and AJ, in cultured mammalian cells exposed to the oxidative insult generated by an array of oxidative stressing agents (H_2O_2, tB-OOH or Ox-LDL) and to compare these results with the antioxidant capacity as assayed with cell-free methods.

4.1. PDERs antioxidant capacity in cell-free systems

In the cell-free experimental setting, PJ, AJ and RE always exhibited a remarkable antioxidant capacity strictly related to their specific polyphenol content. Indeed, in all tests performed the best activity was displayed by RE which contains the highest polyphenol amount (7.48 mg polyphenol ml^{-1}). As to the two juices, the activity of PJ (1.54 mg polyphenol ml^{-1}) was higher than that of AJ (0.57 mg polyphenol ml^{-1}), and this difference is likely to be due to the presence of rind tannins in PJ, as shown by Gil et al. [14] and by Ricci et al. [20]. Indeed rinds contain punicalagin and ellagic acid, which are known as the most potent pomegranate antioxidants, and are also likely to account for RE-afforded cytoprotection (see below).

4.2. PDERs protective effects in oxidatively injured cells

The results we obtained in cultured cells are divergent from those discussed above, and somehow surprising. Indeed, despite the fact that PJ is the most commercially diffused derivative, it never displayed any protective/antioxidant capacity in the two cell lines used throughout this research, namely U937 and HUVEC cells (Figs. 2A–D and 3A and B, respectively). Rather, PJ exerted a slight prooxidant/sensitizing effect in H_2O_2-treated U937 cells (Fig. 2C and D). Similar results were obtained with AJ, whose sensitizing effect was even higher (Fig. 2C). On the contrary, RE (up to 3%, v/v) afforded cytoprotection in all the conditions tested, in both U937 and HUVEC cells (Figs. 2A–D and 3A and B), i.e. in a cell type-independent fashion. Finally, RE, unlike PJ and AJ, also mitigated the cytocidal activity of Ox-LDL in HUVEC cells (Fig. 4). This finding is of utmost importance since a wealth of authoritative reports [12,24,25,29,30,43] have demonstrated that pomegranate preparations (mainly PJ) exert antiatherogenic effects in vivo: notably atherosclerosis is a chronic and degenerative disease where Ox-LDLs play a central etiological role [42,45].

4.3. Role of iron chelation in RE antioxidant capacity

As to the mechanisms of RE-antioxidant effect in cell systems, we have shown that iron chelation might represent a relevant, cooperative (see below) one (Fig. 6A and B) and implicitly, that RE active components, which are capable of hampering tB-OOH-nuclear DNA damage (Fig. 6B), act intracellularly. Iron chelation might depend on the integrity of adjacent oxidizable groups, such as catechol groups of specific polyphenols: indeed, upon shelf storage, which is likely to result in air oxidation of OH-groups, RE looses either its Fe-chelation ability or its cytoprotective potential (Fig. 7). This observation is in agreement with the well established notion that polyphenolic catechol groups may act as antioxidants via a double mechanism, i.e. scavenging and iron-chelating [5,7,46]. It is also important noting that punicalin, ellagic acid and punicalagin isomers, active polyphenols displaying high antioxidant capacity mostly present in rinds, do contain a high *per molecule*-number of adjacent OH-and catechol groups.

4.4. Differential antioxidant capacity of PDERs in cell-free and cellular studies

Taken collectively, our findings indicate that, although all PDERs act as antioxidants in cell-free tests, only RE displays antioxidant capacity in living cells and that its activity apparently seems to be inhibited by the presence of aril components in PJ, which is obtained by juicing both rinds and arils. An explanation for this result may relate to the lower concentration of antioxidant polyphenols in whole PJ (1.54 mg ml^{-1}) as compared with RE (7.48 mg ml^{-1}): indeed the experimental PJ used in this study is composed by a predominant proportion of aril constituents and only a minor of rind ones.

An unanswered question, however, arises from these observations: why do PJ and AJ behave differently in cell-free and cell-based tests? The lower content of specific polyphenols (in particular, punicalin, ellagic acid and punicalagin isomers) of PJ and AJ, as previously mentioned, may explain their lack of cytoprotective capacity, or their lower antioxidant capacity in cell-free systems. From a cellular disposition point of view, one could speculate that PJ and AJ contain concentrations of polyphenols and/or particular active components that are too low for efficient intracellular transport, in the presence of competing inactive compounds. Therefore, the net intracellular accumulation of antioxidant phenolics is below the threshold efficacy limit. On the contrary, in the acellular systems, where no compartmentalization effect takes place, the antioxidant components would act regardless of the presence of the inactive ones.

From a different point of view, it is also worth noting that Seeram et al. [21] found that PJ exerts antiproliferative and proapoptotic activity in human tumour cells and that this effect is higher than that of PJ purified polyphenolic fractions: in this light, although PJ and AJ alone were not toxic under our conditions (up to 3%, v/v, see Section 3), they could slightly sensitize cells to specific harmful conditions such as oxidative stress.

A possible integrative hypothesis might be that, under our treatment conditions, specific aril constituents in PJ become prooxidants in the presence of added oxidants, an event that could counteract the antioxidant capacity of RE components extracted in PJ. Interestingly, Galati et al. [47] reported that specific dietary polyphenolics, in the presence of H_2O_2 and cellular enzymes (myeloperoxidase and peroxidases) are converted to *o*-quinone metabolites. These are capable of oxidizing cellular GSH, an event leading to a perturbed oxidation state and to cell damage. The fact that PJ- and AJ-supplementation of H_2O_2-injured U937 cells resulted in a higher rate of NPSH consumption (Fig. 5) would indirectly lend support to this hypothesis.

It is unlikely that the slight prooxidant PJ activity we found in cells exposed to H_2O_2 (Figs. 2C and 3A) may be toxicologically relevant in vivo, since phenoxyl radicals can be easily quenched by the presence of physiological levels of ascorbic acid [21]. However, the lower antioxidant and cytoprotective capacity of RE upon addition of AJ or of whole PJ, as compared to RE alone, might be detrimental from a chemopreventive point of view.

5. Conclusion

Apart from its use as a table fruit, pomegranate is gaining more attention since its juice, PJ, has been shown to exert potent antiatherogenic effects in vivo [12,24,25,29,45] and this activity has been attributed to its antioxidant property. Our data, however, indicate that RE possesses the highest antioxidant capacity in cell free systems and that only RE, unlike PJ and AJ, shows activity in a selection of cell-based antioxidant assays. For the use of Pomegranate as a nutraceutical to prevent the onset of atherosclerosis, future development should focus on rind-only derivatives. Interestingly, a very recent report by Rosenblat et al. [30] showed that pomegranate by-products (obtained from the husks following juice expulsion) possess far higher antiatherogenic activity as compared to whole PJ. Our data strengthen and extend this finding: in other words avoiding the presence of aril components in rinds preparations should improve their beneficial effects. The preparation of Rosenblat's pomegranate by-products [30] involved pre-squeezing of fruits to obtain PJ followed by husks and rinds processing, i.e. a condition that may cause the absorption of aril components by husks and rinds. It is therefore possible that RE obtained without soaking rind membranes with aril juice (i.e. the method we used to obtain RE) may further enhance its activity. In addition, our preparation does not include the external peel of the fruit, which is more prone to contamination by pesticides and or ambient pollutants.

A technical issue arising from the present work is the extreme susceptibility of RE to air oxidation (Fig. 7) leading to the loss of its antioxidant capacity: this intrinsic problem which is hardly avoidable for liquid preparations, could be opportunely circumvented by developing more stable and affordable derivatives such as dry extracts or lyophilized powders.

As a corollary, and noted by Azzi in a recent review [48], much has to be learnt about the uptake, biotransformation, and tissue distribution of specific molecules (or mixtures of molecules) regularly thought of as "antioxidant" before we conclude that they have such a function in vivo. Our data, although obtained in cultured cells and not in whole organisms, lend support to this view, and imply that the antioxidant capacity of plant-derived preparations from cell-free assays does not necessarily correlate with even simplified living biological systems such as cultured cells. The integration of expeditious and inexpensive cell-free assays with others based on cell culture systems should be recommended as it reveals more information on the actual antioxidant capacity of complex mixtures, such as plant-derived extracts.

Finally, based on our results, it is reasonable to conclude that – to improve the efficacy of PDERs as a nutritional supplement to prevent atherosclerosis in humans – the use of desiccated derivatives based only on the rind might be warranted, due to their superior antioxidant capacity and stability as compared to whole PJ.

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