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# Phenolic-rich juice prevents DNA single-strand breakage and cytotoxicity caused by *tert*-butylhydroperoxide in U937 cells: the role of iron chelation

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**Abstract**

The antioxidant potential of phenolic compounds is generally linked to their ability to scavenge free radicals. However, in addition to their radical-scavenging activity, phenolic compounds can chelate metal ions, such as iron, to prevent their participation in Fenton-type reactions, which lead to the formation of free radicals. The aim of the present study was to evaluate the ability of a phenolic-rich juice made from grapes, cherries and berries to protect human myeloid leukemia (U937) cells from oxidative stress caused by *tert*-butylhydroperoxide (tB-OOH). Preincubation of cells with extracts of the phenolic-rich juice at different concentrations (0–200 μM ferulic acid equivalents) for 3 h partially prevented cell death and abolished the DNA cleavage induced by tB-OOH. Moreover, when preincubating cells with the 100-μM juice extract (the dose that diminished cell death by around 50%), the partial prevention of tB-OOH-induced formation of reactive oxygen species (ROS) and mitochondrial permeability transition pore opening was observed. The radical scavenger antioxidant *N*,*N*′-diphenyl-1,4-phenylene-diamine (DPPD) and the intracellular iron chelator *o*-phenanthroline (*o*-Phe) were also tested to know whether protective effects depended on radical-scavenging or iron-chelating activities. *o*-Phe prevented cell death, DNA cleavage and ROS generation, whereas DPPD only prevented cell death, suggesting that phenolics in the juice afforded protection against induced oxidative stress, most probably by means of an iron-chelating mechanism.


*Keywords:* Phenolics; Iron chelators; Radical scavengers; DNA single-strand breakage; Cytotoxicity; *tert*-Butylhydroperoxide

## 1. Introduction

Several human chronic disease states have been associated with oxidative stress, which occurs in a cell or in a tissue when the concentration of the reactive oxygen species (ROS) generated exceeds the antioxidant capability of that cell [1]. Since the 1990s, different world organizations have recommended an increase in the intake of dietary antioxidants (carotenoids, polyphenols and vitamins C, A and E), with the aim of preventing cancer and many chronic diseases [2,3]. Nowadays, there is growing interest among scientists, food manufacturers and consumers in the study of food properties for maintaining human health [4] — a fact that gives rise to the development of antioxidant-rich functional foods with potential health benefits for consumers. However, any beneficial effect has to be scientifically demonstrated with human intervention studies [5], and functional foods need to be evaluated from a toxicological point of view. For this, in vitro cell culture systems can be used to assess cytotoxicity and cellular responses and to perform toxic kinetic modeling, since they are a valuable tool for elucidating the mechanisms of action of natural antioxidants present in fruit-derived functional foods [6,7]. An experimental in vitro analysis of biological activities of whole foods will therefore serve several purposes. On one hand, the determination of toxicity can be used as a tool to define the concentrations at which chemoprotective effects can be further characterized; on the other hand, cells can be treated with subtoxic concentrations of the compounds to identify new cellular responses, among them mechanisms of potential chemoprevention [7].

Phytochemicals, especially the phenolics found in fruits and vegetables, have been proposed as major bioactive

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compounds that provide health benefits associated with diets rich in plant foods. In line with this, several plant phenolics have been found to show strong antioxidant activity [8–10] and to protect against oxidant-induced damage in cell culture models [11–18].

The principal hypothesis associated with the biological effects of phenolics is linked to their radical-scavenging properties. However, in addition to directly quenching ROS and free radicals, phenolic compounds can chelate metal ions such as iron to prevent their participation in Fenton-type reactions, which lead to the formation of free radicals [8,19]. Although iron chelation has generally been regarded as playing a minor role in the antioxidant activity of polyphenols, the presence of iron-chelating groups in foods and their efficiency in iron chelation may partly explain the health-protective role of specific phenolics in the human diet [20,21]. Nevertheless, it is not easy to establish the relative contribution of the radical-scavenging properties versus the iron-chelating properties of polyphenols to their antioxidant properties.

To address this issue, Sestili et al. [11,15] developed a U937-cell-based approach, which, in a relevant biological setting, discriminates free-radical-scavenging versus iron-chelating mechanisms. The approach is based on the observation that DNA cleavage evoked by the oxidant *tert*-butylhydroperoxide (tB-OOH) is abolished by iron chelators and is insensitive to radical scavenger antioxidants [22–25], whereas the cell death induced by tB-OOH is abolished by both iron chelators and antioxidants [22,25]. Using this experimental approach, they have demonstrated that the most prominent activity of selected flavonoids and hydroxycinnamic acids that affords protection against tB-OOH-induced cell death and DNA damage resides in their ability to chelate iron [11,15]. This fact has been further confirmed by other researchers in a Caco-2 cell model [12].

In the present study, we evaluated the cytoprotective activity of a phenolic-rich juice made from pigmented fruits against tB-OOH-induced oxidative stress in U937 cells. The aim was to find out whether the abovementioned experimental approach is suitable to elucidate the most prominent antioxidant mechanism displayed by a whole phenolic-rich food.

## 2. Materials and methods

### 2.1. Chemicals

tB-OOH, *N*,*N*'-diphenyl-1,4-phenylene-diamine (DPPD) and *o*-phenanthroline (*o*-Phe), as well as most reagent-grade chemicals, were obtained from Sigma-Aldrich (Milan, Italy). Dihydrorhodamine 123 (DHR) and calcein acetoxymethylester (calcein-AM) were from Molecular Probes Europe (Leiden, The Netherlands). Cyclosporin A (CsA) was obtained from Sandoz (Bern, Switzerland). Folin–Ciocalteu's phenol reagent, 2,2-diphenyl-1-picrylhydrazyl assay (DPPH), 2,2′-azinobis(3-ethylbenzothiazoline 6-sulfonate) (ABTS), 6-hydroxy-2,5,7,8-tetramethyl-chroman-2-carboxylic acid (Trolox) and ethylenediaminetetraacetic acid (EDTA) were purchased from Sigma-Aldrich (Madrid, Spain). 2,4,6-Tri-pyridil-*s*-triazine (TPTZ) and ferulic acid were from Fluka (Buchs, Switzerland). Ferrous sulphate heptahydrate ($FeSO_4 \cdot 7H_2O$) was from Panreac (Barcelona, Spain).

### 2.2. Description and analysis of the juice

The experimental juice was prepared by the Research and Development Department of Hero Spain (Alcantarilla, Murcia, Spain). The major ingredient was water, which was mixed with commercially available concentrated juices of grape (26%), cherry (2%), raspberry (1%), blackberry (0.6%) and blackcurrant (0.6%). The total phenolic compounds were analyzed by a colorimetric assay using Folin–Ciocalteu's phenol reagent [26]. Ferulic acid was used as standard, and the total phenolic content is expressed as milligrams per liter or as micromolars of ferulic acid equivalents for cell culture assays.

The main groups of phenolic compounds were analyzed by high-performance liquid chromatography (HPLC) according to Cantos et al. [27]. Briefly, phenolics were extracted with HPLC-grade methanol plus 3% formic acid and analyzed on an L-7100 liquid chromatograph equipped with a Merck-Hitachi 7455 UV diode array detector and a 25×0.4-cm Licrochart RP-18 column with a particle size of 5 mm (Merck, Darmstadt, Germany). The solvents used were water plus 5% formic acid (Solvent A) and HPLC-grade methanol (Solvent B) at a flow rate of 1 ml/min. Elution was performed with a gradient starting with 2% Solvent B to reach 32% Solvent B at 30 min, 40% Solvent B at 40 min and 95% Solvent B at 50 min, which was then isocratic for 5 min. Anthocyanins were quantified at 510 nm, stilbenoids at 320 nm, hydroxycinnamic acid derivatives (caffeic acid derivatives) at 320 nm and catechins at 280 nm.

The total antioxidant activity of the juice was evaluated using four common tests: the Trolox equivalent antioxidant capacity (TEAC; I and II) assay, the DPPH assay and the ferric-reducing/antioxidant power (FRAP) assay. The colorimetric TEAC assay measures the ability of antioxidants to scavenge the ABTS radical cation. The TEAC I assay [28] was carried out using a kit manufactured by Randox Laboratories (Ardmore, UK; cat. no. NX2332). This method is based on antioxidants' inhibition of the absorbance of the radical cations of ABTS at 600 nm. ABTS radical cations are formed by the incubation of ABTS with metmyoglobin (MetMb) and $H_2O_2$. The TEAC II assay [29] is based on the reduction of the radical cation of ABTS, which is generated by filtering an ABTS solution through manganese dioxide powder. The antioxidant activity of the samples is calculated by determining the decrease in absorbance at 734 nm. In the DPPH assay [30], antioxidants reduce the free radical DPPH, and the decrease in absorbance is measured at 515 nm. In all these tests, Trolox was used as standard, and results are expressed as millimoles of Trolox equivalents per liter. The FRAP assay [31] measures the iron-reducing

capacity of the sample to reduce a ferric ($Fe^{3+}$)–TPTZ complex to the ferrous ($Fe^{2+}$) form. Then, an intense blue color develops, which is measured at 593 nm. Aqueous solutions of known $Fe^{2+}$ ($FeSO_4 \cdot 7H_2O$) concentrations were used for calibration. The final results were expressed as millimoles of $Fe^{2+}$ equivalents per liter. One $Fe^{2+}$ equivalent per liter equals the amount of $Fe^{2+}$ per liter required to give the same absorbance change.

### 2.3. Cell culture and treatment

Human myeloid leukemia (U937) cells were cultured in suspension in an RPMI 1640 medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% (vol/vol) fetal bovine serum (Biological Industries, Kibbutz Beit Haemek, Israel), penicillin (50 UI/ml) and streptomycin (50 μg/ml) (Sera-Lab, Crawley Down, UK) at 37 °C in a humidified atmosphere of 95% air–5% $CO_2$. For the experiments, cells ($2.5\times10^5$ cells/ml) were preincubated with extracts of the phenolic-rich juice at different concentrations of total phenolics (0–200 μM) for 3 h in 2 ml of RPMI 1640 medium. The extracts were obtained by directly diluting the juice in RPMI 1640 medium. After being washed, juice-preloaded cells were treated for 15 or 30 min at 37°C in 2 ml of Saline A buffer (8.812 g/L NaCl, 0.372 g/L KCl, 0.336 $NaHCO_3$ and 0.9 g/L glucose) containing tB-OOH. After each treatment, the cells were washed and analyzed immediately for DNA damage, DHR oxidation and calcein staining, or postincubated for cytotoxicity assays. To elucidate the mechanism of action of juice phenolics, we carried out experiments in which cells ($2.5\times10^5$ cells/ml) were preexposed to the radical scavenger antioxidant DPPD (10 μM), the intracellular iron chelator *o*-Phe (25 μM) and the immunosuppressive drug CsA (0.5 μM). These compounds were added to the cultures 5 min prior to the oxidative challenge with tB-OOH in Saline A (2 ml) and assayed as described above. Stock solutions of tB-OOH were freshly prepared in distilled water. DPPD and *o*-Phe were dissolved in dimethyl sulfoxide and CsA in ethanol. At the treatment stage, the final ethanol or dimethyl sulfoxide concentrations were never higher than 0.05%. Under these conditions, ethanol or dimethyl sulfoxide was neither toxic nor DNA-damaging, nor did it affect the cytogenotoxic properties of tB-OOH.

Table 1
Phenolic compounds and total antioxidant activity of the experimental juice

| Parameter | Mean value |
|---|---|
| Phenolic compounds | |
| Total phenols (mg/L ferulic acid equivalents) | 3000 |
| Total phenols (μM ferulic acid equivalents)[a] | 15,000 |
| Anthocyanins (mg/L) | 670 |
| Catechins (mg/L) | 425 |
| Hydroxycinnamic acid (mg/L) | 200 |
| Stilbenoids (mg/L) | 9.5 |
| Total antioxidant activity | |
| TEAC I (ABTS/MetMb) (mM Trolox equivalents) | 25 |
| TEAC II (ABTS/$MnO_2$) (mM Trolox equivalents) | 27 |
| DPPH (mM Trolox equivalents) | 15 |
| FRAP (mM $Fe^{2+}$ equivalents) | 32 |

[a] This value (15,000 μM) is used to adjust the phenolic concentrations for cell culture assays.

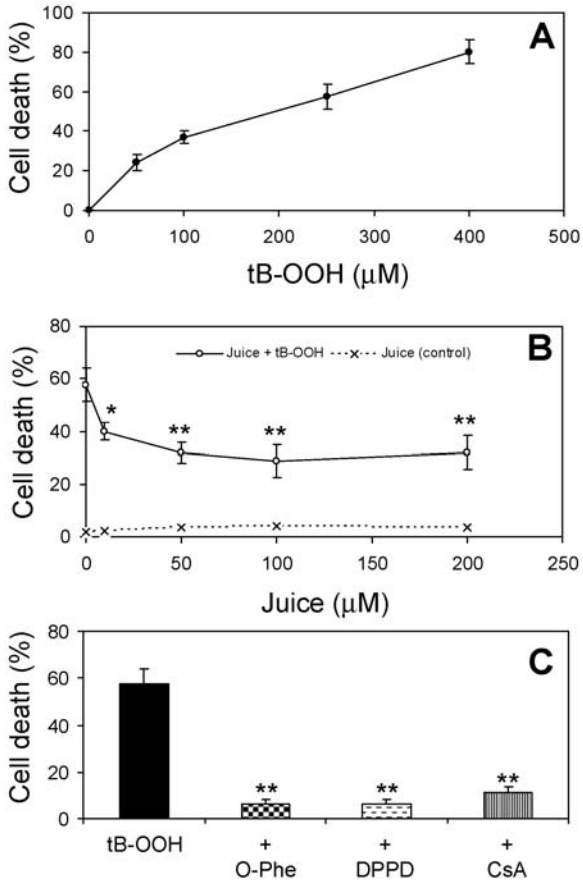


Fig. 1. The effect of the phenolic-rich juice, *o*-Phe, DPPD and CsA on tB-OOH-induced cytotoxicity. (A) Cells challenged with increasing concentrations of tB-OOH. (B) Cells exposed to increasing concentrations of juice extracts and challenged (continuous line) or not (dashed line) with 250 μM tB-OOH. (C) Cells preexposed to 25 μM *o*-Phe, 10 μM DPPD and 0.5 μM CsA and challenged with 250 μM tB-OOH. Results are presented as the mean±S.E.M. of at least three separate experiments. $*P<.05$, $**P<.01$ compared with control cells exposed to tB-OOH (unpaired *t* test).

### 2.4. Cytotoxicity assay

After treatment with tB-OOH for 30 min, the cells were washed with Saline A and resuspended in prewarmed RPMI 1640 medium (2 ml) before being plated into 60-mm tissue culture dishes and incubated at 37°C for 4 h in a fresh culture medium. Cytotoxicity was determined with the trypan blue exclusion assay. Briefly, an aliquot of the cell suspension was diluted 1:1 (vol/vol) with 0.4% trypan blue (Sigma), and the cells were counted with a hemocytometer. The results are expressed as the percentages of dead cells (ratio of stained cells to the total number of cells).

### 2.5. Measurement of DNA single-strand breakage

After treatment with tB-OOH for 30 min, the cells were analyzed immediately for DNA single-strand breakage using the alkaline halo assay [32], with minor modifications. After

treatment, the cells were resuspended ($2.0\times10^4$ per 100 μL) in a 1.5% low-melting-point agarose in phosphate buffer containing 5 mM EDTA and immediately sandwiched between an agarose-coated slide and a coverslip. After complete gelling, coverslips were removed and slides were immersed in an alkaline buffer (0.1 M NaOH/1 mM EDTA, pH 12.5), washed and stained for 5 min with 10 μg/ml ethidium bromide. The ethidium bromide-labeled DNA was visualized using a BX-51 microscope (Olympus, Tokyo, Japan) equipped with a SPOT-RT camera unit (Diagnostic Instruments). The resulting images were digitally acquired and processed at the single-cell level on a personal computer using Scion Image software (Scion, Frederick, MA, USA). DNA single-strand breakage was quantified by calculating the nuclear spreading factor value, which represents the ratio between the area of the halo (obtained by subtracting the area of the nucleus from the total area, nucleus plus halo) and that of the nucleus (from 50 to 75 randomly selected cells/experiment/treatment condition). The results are expressed as relative nuclear spreading factor values calculated by subtracting the nuclear spreading factor values of control cells from those of treated cells.

### 2.6. DHR oxidation, calcein staining and imaging

After treatment with tB-OOH for 15 min, the cells were analyzed immediately for the formation of ROS using the

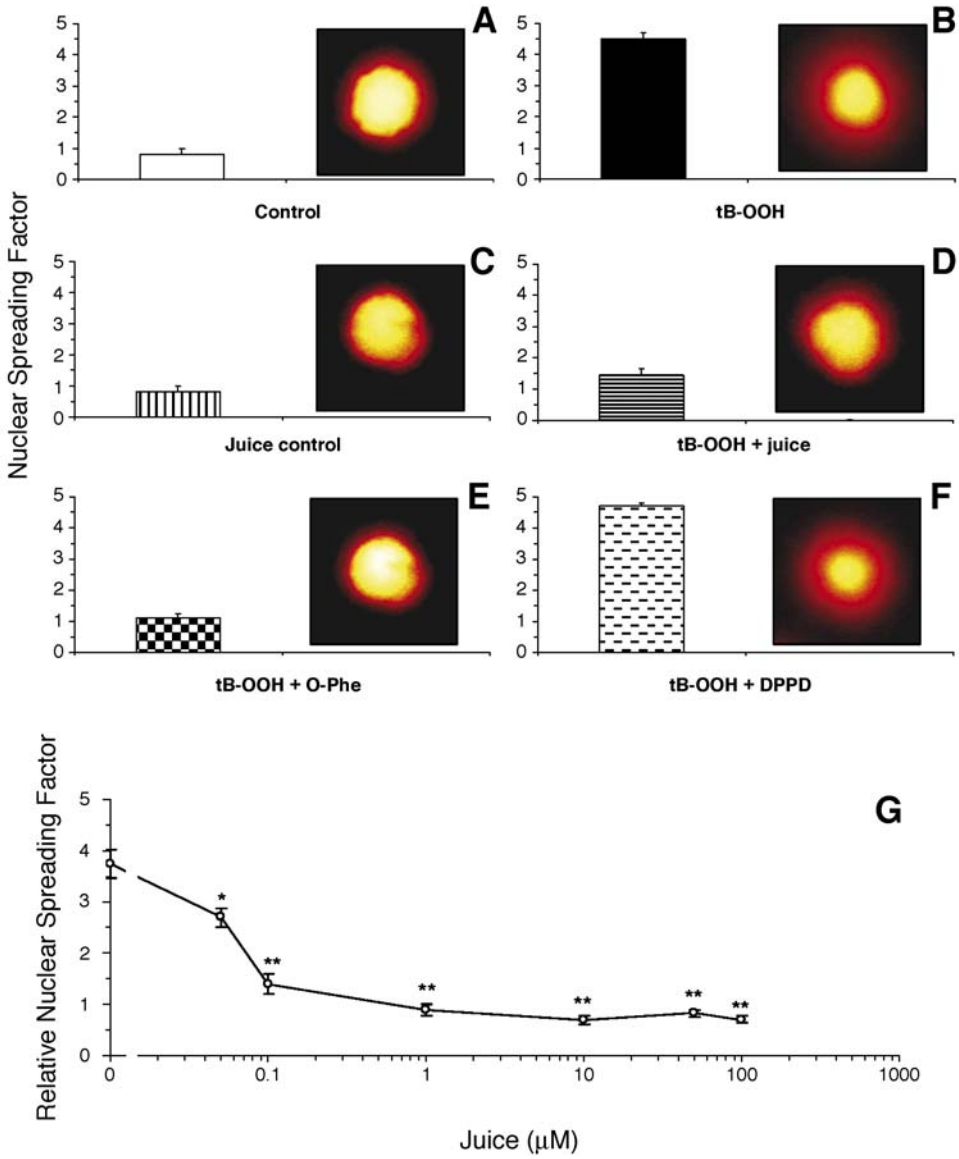


Fig. 2. The effect of the phenolic-rich juice, *o*-Phe and DPPD on tB-OOH-induced DNA single-strand breakage. (A–F) Representative photomicrographs and nuclear spreading factors of U937 cells under the experimental conditions detailed as follows: (A) control cells; (B) cells exposed to 250 μM tB-OOH; (C) cells preloaded with 10 μM juice (juice control); (D) cells preloaded with 10 μM juice that were exposed to 250 μM tB-OOH; (E and F) cells pretreated with 25 μM *o*-Phe and 10 μM DPPD, respectively, and later exposed to 250 μM tB-OOH; (G) cells preincubated with increasing concentrations of juice and exposed to 250 μM tB-OOH. Results are expressed as the relative nuclear spreading factor and represent the mean±S.E.M. of at least three separate experiments. *$P<.05$, **$P<.01$ compared with control cells exposed to tB-OOH (unpaired *t* test).

fluorescent probe DHR [33] and for mitochondrial permeability transition (MPT) pore opening [34]. Cells ($2.5\times10^5$ $ml^{-1}$) were pretreated for 15 min with 250 μM tB-OOH and then postincubated for 10 min in the presence of 10 μM DHR or 1 μM calcein-AM and 1 mM $CoCl_2$. After washing thrice, the cells were resuspended in 20 μL of Saline A and stratified on a slide. Fluorescence images were captured with a BX-51 microscope (Olympus) equipped with a SPOT-RT camera unit (Diagnostic Instruments). The excitation and emission wavelengths were 488 and 515 nm, respectively, with a 5-nm slit width for both emission and excitation. Images were collected with exposure times of 100–400 ms, digitally acquired and processed for fluorescence determination at the single-cell level on a personal computer using Scion Image software (Scion). Mean fluorescence values were determined by averaging the fluorescence values of at least 50 cells/treatment/experiment.

### 2.7. Statistical analysis

Data were analyzed by Statistical Package SPSS, version 11.5, for Windows (SPSS, Chicago, IL, USA). Results are expressed as mean±S.E.M. For comparisons between two groups, Student's unpaired *t* test was used. $P<.05$ was considered as the level of statistical significance. *P* values between .01 and .05 are presented in the figures.

## 3. Results

### 3.1. Antioxidant properties of the juice

Table 1 shows the phenolic compounds and antioxidant properties of the experimental juice. As might be expected from the product design, the juice showed a high total amount of phenolics and a strong antioxidant activity in terms of iron-reducing capacity and the scavenging of free radicals in different in vitro tests. The main phenolics were anthocyanins, followed by catechins, hydroxycinnamic acids (caffeic acid derivatives) and stilbenoids. As regards the antioxidant properties of the juice, this product showed an antioxidant activity higher than those of many fresh fruits and vegetables — even higher than those of beverages recognized as important antioxidants in the diet (tea, coffee and beer) — and similar to that of red wine [4]. These results are in accordance with those observed in a previous study, in which we evaluated the antioxidant properties of a jellified dessert designed as a functional food and prepared using the same formulation [35].

### 3.2. Phenolic-rich juice prevents tB-OOH-induced cytotoxicity

As shown in Fig. 1A, treatment with increasing concentrations of tB-OOH for 30 min, followed by posttreatment incubation for 4 h in a fresh culture medium, increased cell death in a dose-dependent manner. Conditions were chosen so that 30 min of incubation at a tB-OOH concentration of 250 μM decreases cell viability by about 60%. As can be seen from Fig. 1B, the cytotoxic effect of tB-OOH was partially mitigated in cells preincubated with juice extracts at increasing concentrations, reaching a plateau when cells were preincubated with juice extracts at a phenolic concentration of 50 μM or above (Fig. 1B, continuous line). Importantly, juice extracts alone did not promote cytotoxicity at any of the concentrations tested, and cell death was always <5% throughout the test period (Fig. 1B, dashed line). As illustrated in Fig. 1C, under similar experimental conditions, the radical scavenger antioxidant DPPD (10 μM) and the intracellular iron chelator *o*-Phe (25 μM) also promoted cytoprotection. In addition, cells were rescued by the MPT inhibitor CsA (0.5 μM) [36].

### 3.3. Phenolic-rich juice prevents DNA single-strand breakage induced by tB-OOH

Fig. 2 shows the effect of preincubation with extracts of the phenolic-rich juice on DNA cleavage produced by tB-OOH. Preincubation of cells with juice extracts at increasing concentrations for 3 h prevented DNA strand scission caused by tB-OOH. Fig. 2A–F shows nuclear spreading factors and representative photomicrographs of ethidium-

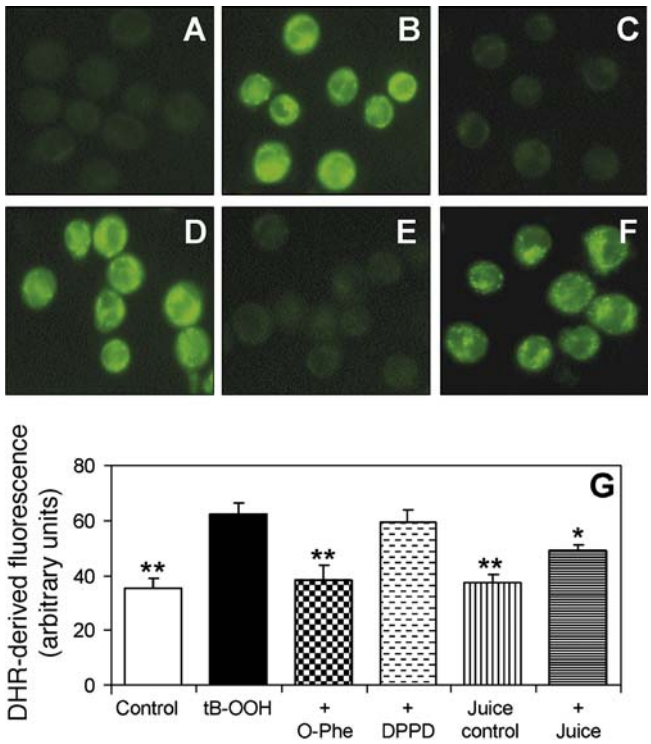


Fig. 3. The effect of the phenolic-rich juice, *o*-Phe and DPPD on tB-OOH-induced DHR-derived fluorescence. (A–F) Representative photomicrographs of U937 cells under different experimental conditions. (A) Control cells; (B) cells exposed to 250 μM tB-OOH; (C and D) cells preexposed to 25 μM *o*-Phe and 10 μM DPPD, respectively, and later challenged with 250 μM tB-OOH; (E) cells preloaded with 100 μM juice (juice control); (F) cells preloaded with 100 μM juice and exposed to 250 μM tB-OOH; (G) quantification of DHR-derived fluorescence under the experimental conditions described above. Results represent the mean±S.E.M. of at least three separate experiments. $*P<.05$, $**P<.01$ compared with control cells exposed to tB-OOH (unpaired *t* test).

bromide-stained nuclei. As shown in Fig. 2B, treatment with 250 μM tB-OOH for 30 min was highly DNA-damaging and, consequently, led to an increase in the nuclear spreading factor compared to that observed in untreated control cells (Fig. 2A). As shown in Fig. 2D, preincubation with the 10-μM juice extract virtually abolished the extensive DNA cleavage caused by tB-OOH. As illustrated in Fig. 2C, the 10-μM juice extract alone (juice control) was not DNA-damaging and showed a nuclear spreading factor similar to that of untreated control cells. Fig. 2E demonstrates that the intracellular iron chelator *o*-Phe (25 μM) abolished tB-OOH-induced DNA cleavage, while in Fig. 2F, it can be seen that an antioxidant such as DPPD (10 μM) was not able to protect against such cleavage. As illustrated in Fig. 2G, preincubation of cells with extracts of the phenolic-rich juice at increasing concentrations for 3 h effectively reduced tB-OOH-induced DNA strand scission in a dose-dependent manner. A plateau was reached when cells were preincubated with juice extracts at a phenolic concentration of 10 μM or above. Moreover, juice extracts alone were not DNA-damaging at any of the concentrations tested (data not shown).

### *3.4. Phenolic-rich juice prevents tB-OOH-induced intracellular ROS generation*

Fig. 3 shows the effect of the phenolic-rich juice, *o*-Phe and DPPD on tB-OOH-induced intracellular ROS generation. The results shown in the bar graph in Fig. 3G are illustrated in (A)–(F). Fig. 3B shows a representative photomicrograph of cells exposed to 250 μM tB-OOH in which the fluorescence is much stronger than in the control cells of Fig. 3A. Exposure to tB-OOH significantly increased intracellular oxidative stress and led to an increase in DHR-derived fluorescence due to DHR oxidation. As shown in Fig. 3C, the addition of the iron chelator *o*-Phe (25 μM) to the cultures inhibited the formation of ROS, giving a fluorescence intensity similar to that of control cells. However, as shown in Fig. 3D, the antioxidant DPPD (10 μM) did not inhibit intracellular oxidative stress and the observed DHR-derived fluorescence was similar to that of cells exposed to tB-OOH. As illustrated in Fig. 3F, the preincubation of cells with the 100-μM juice extract for 3 h partially inhibited the formation of ROS, leading to a lower fluorescence intensity after exposure to tB-OOH. Importantly, as shown in Fig. 3E, the juice extract alone did not increase the formation of ROS and a gave fluorescence intensity similar to that of control cells.

### *3.5. Phenolic-rich juice prevents tB-OOH-induced MPT pore opening*

Fig. 4 shows the effect of the phenolic-rich juice and CsA on tB-OOH-induced MTP pore opening in U937 cells. MPT pore opening was assessed by monitoring the changes in mitochondrial calcein fluorescence after quenching cytosolic and nuclear signals with $Co^{2+}$ [34]. As illustrated in Fig. 4A, the resulting punctuate fluorescence was caused by the calcein localized in the mitochondrial compartment. As can be seen from Fig. 4B, the loss of mitochondrial calcein therefore acts as a strong indication of MPT pore opening. This event was clearly detected in cells exposed to 250 μM tB-OOH for 15 min but was not observed in control cells (Fig. 4A). The image shown in Fig. 4B should be compared to that shown in Fig. 4C, in which the presence of the cells is demonstrated by darkening the digital image. As illustrated in Fig. 4D, MPT pore opening was fully prevented by the MPT inhibitor CsA (0.5 μM). As shown in Fig. 4E, preincubation of cells with the 100-μM juice extract alone (juice control) did not cause MPT pore

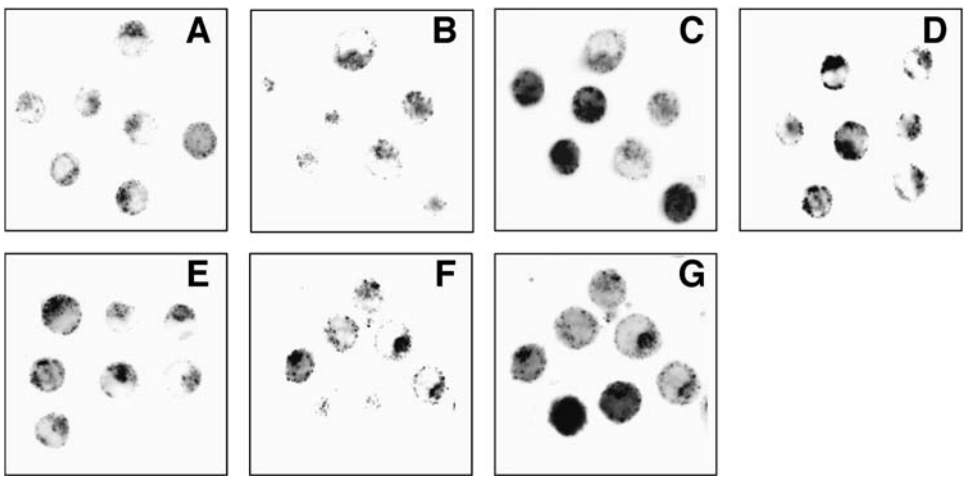


Fig. 4. The effect of the phenolic-rich juice and CsA on tB-OOH-induced MPT pore opening. Representative photomicrographs of U937 cells pretreated or not for 15 min with 250 μM tB-OOH in the absence or presence of juice or CsA and then postincubated for 10 min in the presence of 1 μM calcein-AM and 1 mM $CoCl_2$. (A) Control cells; (B) cells exposed to 250 μM tB-OOH; (D) cells exposed to 250 μM tB-OOH in the presence of 0.5 μM CsA; (E) cells preloaded with 100 μM juice (juice control); (F) cells preloaded with 100 μM juice and exposed to 250 μM tB-OOH; (C) and (G) same images as (B) and (F), respectively, in which the presence of cells is demonstrated by darkening the digital image. The micrographs are representative of at least three separate experiments with similar outcomes.

opening, while MPT pore opening was partially prevented in the cells preloaded with 100 μM juice and exposed to tB-OOH (Fig. 4F). The image shown in Fig. 4F should be compared to that shown in Fig. 4G, in which the presence of the cells is demonstrated by darkening the digital image.

## 4. Discussion

In the present study, U937 cells were challenged with tB-OOH, which is a well-characterized model compound for the study of the mechanisms of oxidative cell injury. The organic hydroperoxide tB-OOH induces an array of cellular dysfunctions, including peroxidation of membrane lipids, depletion of GSH, perturbation of calcium ion sequestration, DNA single-strand breakage and mitochondrial damage [25,37]. The above results show that preincubation of cells with extracts of the phenolic-rich juice designed as a functional food at increasing concentrations for 3 h partially prevented cell death and abolished the DNA cleavage induced by tB-OOH. A similar behavior has been reported in U937 cells challenged with tB-OOH, where quercetin (50 μg/ml), as well as catechin and epicatechin (100 μg/ml), almost fully abolished the DNA cleavage, but only prevented cell death by around 50% [18]. This suggests that, when tested at equal concentrations, phenolics are more effective in preventing DNA cleavage than in preventing cytotoxicity.

It should be noted that, in our study, the protection afforded by the juice was not dose-dependent at concentrations of ≥10 or ≥50 μM for DNA damage and cell death, respectively. In line with this, in a previous study in which we tested the same juice at the same concentrations, we observed a partial and dose-independent prevention of both tB-OOH-induced cell death and lipid peroxidation in HepG2 cells [38], and similar trends have been reported in other cell-based studies. For example, in HL-60 cells, quercetin was reported to prevent menadione-induced ROS and $O_2^-$ generation in a dose-dependent fashion at concentrations up to 25 μM but later approached a plateau when concentrations of ≥50 μM were tested. The same was observed for DNA damage, as concentrations of 10 and 100 μM quercetin achieved the same protection [13]. Similarly, the partial prevention afforded by the flavonoids rutin and quercetin against tB-OOH-induced lipid peroxidation in red blood cells also remained similar at concentrations of ≥100 μM [16]. Furthermore, in a toxicity paradigm in which HepG2 cells were challenged with $Pb^{2+}$, epigallocatechin was shown to achieve a maximum protection at 40–50 μM, while the cytoprotective effect of epicatechin gallate reached a plateau at concentrations of ≥3 μM [14].

These data indicate that, when a given concentration is reached, some types of phenolics do not further protect against oxidative damage. This could be explained by differences in the cellular uptake of different phenolics since molecular features (e.g., lipophilicity, nature of sugar moieties and number of OH groups) and/or other food matrix components (e.g., sugars and acids) may affect polyphenol uptake and, thus, the protection afforded by these phytochemicals. In this regard, it has been reported that quercetin and quercetin-3-glucoside uptake by Caco-2 cells positively correlated with concentrations up to a certain maximal dosage (40 and 100 nM, respectively) [39]. Also in Caco-2 cells, epigallocatechin gallate uptake approached a plateau at concentrations of ≥100 μM, indicating saturable uptake [40]. These observations suggest that, under certain experimental conditions, the cellular uptake of phenolics might depend on a saturable process and, therefore, it could be hypothesized that such a fact might have contributed to the lack of dose dependency observed in our study.

Although the product in question showed high antioxidant activity, it is unclear whether the protective effects exerted by the phenolic-rich juice are dependent on radical-scavenging or iron-chelating activities. In addition to their redox properties, polyphenols have a metal chelation potential that may decrease oxygen toxicity in cells, since transition metals such as iron can participate in the generation of ROS [8,19]. It is generally thought that the following functional groups are important for iron chelation: (a) *ortho*-dihydroxyl groups (e.g., 3′–4′ and 7–8 dihydroxyl groups); (b) the presence of 5-OH and/or 3-OH in conjunction with a C4 keto group (e.g., quercetin); and (c) a large number of OH groups (e.g., tannic acid) [41]. Of these, the presence of two hydroxyl groups in the *o*-position has been reported to be critical for the chelation of iron and the protection afforded by phenolic compounds [11,15]. This essential structural feature is shared by flavonoids (e.g., catechins and anthocyanins) and hydroxycinnamic acids, which were well represented in the juice tested (Table 1). Therefore, since these polyphenols possess ideal structural features for iron chelation, it is reasonable to think that the protective effects exerted by the juice might partly be explained by iron-chelating activity. To address this question, the radical scavenger antioxidant DPPD and the intracellular iron chelator *o*-Phe were also tested.

As mentioned before, our experimental approach is based on the notion that iron chelators suppress DNA strand scission and the cytotoxicity induced by tB-OOH, whereas radical scavenger antioxidants prevent only the latter response. In this context, tB-OOH-induced cell death and DNA damage are proposed to depend on an intracellular source of iron that is able to catalyze the formation of potent oxidizing species by Fenton reaction ($tB\text{-}OOH+Fe^{2+}\rightarrow tBO^{\cdot}+OH^-+Fe^{3+}$) [22,24]. For this reason, iron chelators such as *o*-Phe or desferroxamine [11,12,15,22,24,42], as well as phenolic compounds showing iron-chelating activity [11,12,15], are effective in preventing both cell death and DNA cleavage provoked by tB-OOH.

On the other hand, the fact that antioxidants such as DPPD, Trolox or butylated hydroxytoluene abolish cell death without preventing the formation of DNA lesions [11,12,15,22,24] suggests that the species involved in cytotoxic and genotoxic responses are different. That is,

cell death and the accumulation of DNA damage are not interconnected [22,24,25]. In addition, tB-OOH-induced cell death has been linked to iron-catalyzed lipid peroxidation, but not to DNA damage [11,22,24,25]. This may explain why the chain-breaking antioxidant DPPD, unlike *o*-Phe, prevents cell death but not DNA cleavage and, therefore, allows discrimination between radical-scavenging and iron-chelating activities. In line with this, our results suggest that the most prominent activity of phenolics in the functional juice resides in their ability to chelate iron, since both tB-OOH-induced cell death and DNA cleavage were prevented by juice extracts and *o*-Phe, while DPPD only prevented cell death.

Moreover, after preincubating the cells with the 100-μM juice extract (the dose that diminished cell death by around 50%), partial prevention of tB-OOH-induced MPT opening and intracellular generation of ROS were observed. The formation of ROS was assessed as a measure of intracellular oxidative stress using the fluorescent probe DHR, which accumulates preferentially in the mitochondria and fluoresces when oxidized by various ROS, including $H_2O_2$ [43,44]. Although DHR also accumulates in cell compartments other than the mitochondria (e.g., lysosomes, peroxisomes or endoplasmic reticulum), this fluorescent probe is considered a useful marker of ROS generation at the mitochondrial level [44]. The mitochondrial generation of ROS has been linked to the accumulation of DNA lesions in U937 cells exposed to tB-OOH in a process that is modulated by $Ca^{2+}$. In brief, exposure to tB-OOH elevates intramitochondrial $Ca^{2+}$ concentration, thereby stimulating the mitochondria-generation reactive species (e.g., superoxides and mainly $H_2O_2$), which mediate part of the DNA damage caused by tB-OOH likely via further interaction with $Fe^{2+}$ [25,45]. Importantly, when these authors treated cells with a combination of caffeine (an agent that increases mitochondrial $Ca^{2+}$ uptake) and tB-OOH, DNA damage was markedly increased and, more importantly, this enhanced DNA damage was also prevented by iron chelators and was insensitive to radical scavenger antioxidants [45]. Therefore, our data on the inhibition of ROS generation and the prevention of DNA cleavage also suggest that the most prominent activity of phenolics in the juice is iron chelation, since we observed that such events were prevented by both juice and *o*-Phe, but not by DPPD.

Finally, to further characterize the protection afforded by the phenolic-rich juice, we assessed the effect on MPT pore opening. MPT is a $Ca^{2+}$-induced, nonselective, inner mitochondrial membrane permeabilization process that results in the loss of matrix components, impairment of mitochondrial functionality and substantial swelling of organelles, with consequent outer membrane rupture and cytochrome *c* release, eventually leading to cell death. The molecular mechanism of MPT pore opening appears to involve $Ca^{2+}$-triggered modification and the assembly of various inner and outer mitochondrial membrane components, including the ADP/ATP translocator, cyclophilin D and, possibly, porin and hexokinase [46]. The oxidative stress induced by tB-OOH treatment has been reported to increase cyclophilin D binding to the inner mitochondrial membrane, at the same time increasing the sensitivity of pore opening to $Ca^{2+}$. In this regard, CsA prevents MPT-dependent toxicity by binding to cyclophilin D, thereby specifically blocking MPT pore opening [36,47]. Moreover, the occurrence of MPT pore opening has been linked to an increase in the mitochondrial generation of ROS, including $H_2O_2$. Particularly, after exposure to tB-OOH, increased mitochondrial $Ca^{2+}$ stimulates intramitochondrial ROS generation, which, in turn, initiates MPT pore opening [48,49]. Therefore, it is reasonable to think that MPT-dependent cell death could be prevented by antioxidants. In this study, we observed the partial prevention of both cell death and MPT pore opening after preincubation with the juice. This suggests that, unlike CsA, the cytoprotective effect of the juice might be related to its ability to prevent events prior to the opening of MPT pores, particularly at the ROS generation stage.

In conclusion, under the described experimental conditions, the functional juice under study afforded protection against induced cytotoxicity, DNA cleavage, MPT pore opening and intracellular ROS generation. This suggests that phenolics from the juice were, to some extent, taken up by cells and contributed to their antioxidant defenses. Moreover, on the basis of a well-defined experimental approach, the possible role of an iron-chelating mechanism, potentially of biological relevance, is proposed as being partially responsible for these protective effects. Last but not the least, the possibility that juice phenolics had exerted their protective effects extracellularly by triggering signal transduction cascades within the cell should not be ruled out. For this reason, further studies are needed to find out the relative contribution of extracellular and intracellular effects exerted by antioxidant phytochemicals from whole foods, as their efficacy could largely depend on synergistic effects.

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