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Polyphenols of peanut (*Arachis hypogaea* L.) skin as bioprotectors of normal cells. Studies of cytotoxicity, cytoprotection and interaction with ROS

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ABSTRACT

Arachis hypogaea L. (peanut) skin is a potential source of natural antioxidants, and several studies have suggested the possibility of producing functional ingredients from this by-product of food industry. This investigation examined possible toxic effects of peanut skin polyphenolic extract (PSE) and its antioxidant properties using *in vitro* studies. PSE and its main component quercetin (QE) did not present any cytotoxicity on normal epithelial cells, rat ileum cells (IEC-18), monkey kidney cells (Vero) or human peripheral blood mononuclear cells (PBMCs) at concentrations with antioxidant effects. QE and PSE showed scavenging of the superoxide anion radical and cytoprotection, as well as reducing the reactive oxygen species (ROS) and superoxide dismutase (SOD) activity in IEC-18 cells against menadione-induced oxidative stress. This suggests that peanut skin phenolic extract could be a potential functional ingredient for foods.

1. Introduction

In recent years, there have been important changes in dietary habits with an increasing demand for healthy foods that can add health benefits beyond basic nutrition (Arya, Salve, & Chauhan, 2016). This has led to a growing interest in the so-called functional foods, i.e. food products that can produce positive health effects and decrease the risk of developing certain diseases (Herrero, Cifuentes, & Ibañez, 2006). These changes, in turn, have encouraged industries to improve food formulations and thereby ensure better food safety (Munekata, Fernandes, de Melo, Trindade, & Lorenzo, 2016). This interest in functional foods has also fostered research on new natural sources of bioactive ingredients (Pepe et al., 2016). Of these, vegetables are by far the most studied natural sources, since they contain a huge variety of bioactive compounds that could be used in the food industry as functional ingredients or nutraceuticals (Ugartondo, Touriño, Torres, & Vinardell, 2007; Vijayalaxmi, Jayalakshmi, & Sreeramulu, 2015).

Peanut is an important grain legume cultivated worldwide. Although its uses vary from country to country, more than 50% of the overall peanut world production is crushed for oil and peanut butter production, with the extraction of peanut oil being commonly performed from roasted and/or blanched (i.e. without seed coat or skin) seeds. The peanut skin is a by-product of blanching operations, but its only current market is low value animal feed applications. However, several studies have suggested the possibility of producing functional ingredients from peanut skin, as this material is a very rich source of several phenolic compounds, including phenolic acids, flavonoids, stilbenes and various procyanidin and proanthocyanidin oligomers (Bodoira, Rossi, Montenegro, Maestri, & Velez, 2017; Larrauri, Zunino, Zygadlo, Grosso, & Nepote, 2016; Nepote, Grosso, & Guzmán, 2005; Oldoni et al., 2016). Argentina is the major producer and exporter of peanuts in Latin America, with the production of blanched peanuts generating an estimated 15.000 tons of skin per year.

Various studies have reported antioxidant properties of peanut skin phenolics, mainly due to their scavenging ability against peroxyl and other common free radicals (Ballard, Mallikarjunan, Zhou, & Keefe, 2010; Bodoira et al., 2017; Larrauri et al., 2016; Oldoni et al., 2016). Moreover, according to Francisco and Resurreción (2008), phenolics from peanut skin could have a higher peroxyl-radical scavenging capacity compared with other well-known recognized sources of natural

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phenolics, such as green tea, blueberries and blackberries.

Owing mainly to their scavenging properties against reactive oxygen species (ROS), peanut skin phenolics might have potent antioxidant capacities in both food and biological systems. For instance, peanut skin extracts were found to have protective effects against oxidative degradation of sunflower oil (Larrauri et al., 2016) and a Fe^{2+} ions chelating capacity when used for preservation of sheep patties (Munekata et al., 2016). Moreover, they showed good antioxidant activity in oil-in-water emulsions (Bodoira et al., 2017). Interestingly, catechin and epicatechin derivatives, which are present in peanut skins, (Sarnoski, Johnson, Reed, Tanko, & Keefe, 2012) have been associated with inflammation mitigation by reducing the extent of the synthesis of inflammatory mediator molecules (Atanasov et al., 2018).

In summary, the peanut skin, an undervalued by-product of peanut oil, butter and snack industries, is in fact an important source of bioactive and nutraceutical compounds. In a previous study (Bodoira et al., 2017), we were able to obtain peanut skin phenolic extracts (PSE) with potent antioxidant capacities by means of pressurized liquid extraction (PLE), using two generally recognized as safe (GRAS) solvents (water and ethanol). In the present investigation, we assess whether these PSE, with known phenolic compositions, have cytotoxicity and/or bioprotection effects against oxidative stress in normal cells.

Phenolic compounds from several plant sources have shown diverse *in vitro* results, including cytoprotective (Da et al., 2013) and neuroprotective (González-Sarrías, Núñez-Sánchez, Tomás-Barberán, & Espín, 2017) effects, the promotion or inhibition of iron uptake in Caco-2 cells (Hart, Tako, & Glahn, 2017), and the reduction of lipid accumulation in oleic acid-treated HepG2 cells (Wu et al., 2014), among others.

In the present study, the possible cytotoxic effects of PSE were evaluated in a line of monkey kidney epithelial cells (Vero) and rat ileal epithelial cells (IEC-18). These are normal cell lines, but they have not been previously used in models for evaluating the toxicity of polyphenols. In addition, PSE was assayed on PBMCs, which have been used in models to detect any potential risk to human health (Shanna et al., 2017; Valent, Pereira, Andrade, & Figueiredo-gonz, 2017). The generation of ROS, activity of SOD and the scavenging of the superoxide anion radical were investigated with the aim of determining the protective effects of the bioactive compounds. Finally, the bioprotection effects of PSE were tested against menadione (MEN), an oxidative stress-inducer (Aherne & O'Brien Nora, 2000), on IEC-18 cells, a cell line which can be used as a model for testing absorption at the intestinal level (Steensma, Noteborn, & Kuiper, 2004). In all cases, quercetin (QE), a well-known bioactive flavonoid (Hajihashemi & Geuns, 2013; Vanden Braber et al., 2018), was used as a reference compound.

2. Material and methods

2.1. Materials and chemicals

Phosphate buffered saline (PBS) and trypsin–EDTA (0.05%) were purchased from Gibco (Invitrogen, Grand Island, NY, USA). Neutral red (NR), 1-(4,5-dimethylthiazol-2-yl)-3,5-diphenyltetrazolium bromide (MTT), quercetin (QE), menadione (2-methyl-1,4-naphtoquinone sodium bisulfite, purity: 95%), trypan blue dye (TB), hystopaque®-1077, hydroethidine (HE), nitroblue tetrazolium (NBT), superoxide dismutase (SOD), 2-mercaptoethanol, and hydroxylamine hydrochloride (HAHC) were purchased from Sigma Aldrich (St. Louis, MO, USA). Ethylenediaminetetraacetic (EDTA) was provided by Merck (Germany); Dimethyl sulfoxide (DMSO) was obtained from Sintorgan (Buenos Aires, Argentina).

2.2. Preparation of PSE

Peanut skins were obtained from runner-type peanuts by means of a typical industrial blanching process (90 °C, 10 min). This material was milled and sieved to obtain a uniform particle size (mean value 0.5 mm), and then extracted by means of PLE using an in-house developed apparatus according to an experimental setup reported previously by Bodoira et al. (2017). Briefly, the extraction conditions (temperature, solvent mass flow and pressure) were fixed at 220 °C, 7 g/min and 7 MPa, respectively, with distilled water and absolute ethanol (40:60, v/v) used as the solvent. After the extraction process, the solution obtained was concentrated under vacuum (40 °C) and the residue was dried by lyophilization (Pfeiffer, Mod: DUO 5 M, Germany). The dry extract (hereafter PSE) was stored in an amber glass container under nitrogen at −20 °C until use. The phenolic profile and selected antioxidant properties of the PSE were described in a previous paper (Bodoira et al., 2017). Twenty-four phenolic compounds were identified in PSE by the HPLC-ESI – MS/MS analysis, with the polyphenol pattern found in the PSE being dominated by procyanidin dimers (approx. 75%), monomeric flavonoids including catechin (4.31%), epicatechin (3.53%), quercetin (2.09%) and biochanin A (1.82%), among others, and by proanthocyanidin dimers (approx. 5%).

2.3. Cell culture

The IEC-18 cells (Quaroni & Kurt, 1981) were obtained from the American Type Culture Collection (ATCC, USA). These were maintained in Dulbecco's Modified Eagle Medium (DMEM; Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS; Natocor, Carlos Paz, Argentina), 100 U/mL penicillin-100 μg/mL, streptomycin and 1/100 CTS™ GlutaMAX™-I Supplement (Gibco, Grand Island, NY, USA), at 37 °C in a humidified atmosphere of 5% CO_2 (Arata et al., 2019).

The Vero cell line C76-ATCC (African green monkey kidney epithelial cells) (Yasumura & Kawakita, 1963) was obtained from Asociación Banco Argentino de Células (ABAC, Buenos Aires, Argentina). These cells were cultured in Eagle's minimal essential medium (EMEM; Gibco, Grand Island, NY, USA) supplemented with 8% FBS, gentamycin 50 μg/mL and 2 mM glutamine (Sigma St. Louis, USA), and incubated at 37 °C with 5% CO_2 and a relative humidity of about 95 percent (Escobar et al., 2012).

Human PBMCs were isolated using Hystopaque®-1077 (Sigma Aldrich; St. Louis, MO, USA) from peripheral blood of healthy volunteers (23–25 years old), with informed consent being obtained from each participant. These cells were cultured in Roswell Park Memorial Institute (RPMI)-1640 medium, supplemented with 25 mM Hepes, 2 mM L-glutamine, 50 mM 2-mercaptoethanol, 100 μg/mL streptomycin, 100 μg/mL penicillin (Sigma Aldrich; St. Louis, MO, USA) and 5% FBS. This system was incubated at 37 °C with 5% CO_2 and humidity (Cariddi et al., 2015).

2.4. Cytotoxicity assays

For *in vitro* experiments, PSE and QE were solubilized in DMSO ($\leq$0.05% in the culture medium), with control cells being tested in parallel and submitted to the same treatment in medium with 0.05% DMSO.

The cellular viability of IEC-18 cells was studied by the MTT method (Mosmann, 1983), as previously described by Arata et al. (2019). IEC-18 cells were seeded at 5×10^4 cells/well in 96-well plates, grown for 24 h, and exposed to different concentrations of PSE or QE (concentration range 31–500 μg/mL) for 24 h. Then, the medium was discarded and replaced with DMEM without FBS, but containing 0.5 mg/mL of MTT. The plates were incubated for 4 h at 37 °C in darkness, and the MTT solution was discarded and the blue crystals were solubilized with DMSO. The absorbance was read at 570 nm, using the microplate spectrophotometer reader Multiskan GO (Thermo Fisher Scientific), which was directly proportional to the production of formazan and represented the viable cells.

Vero cell viability was measured by the neutral red uptake assay

(NRU) (Borenfreund & Puerner, 1985), as described previously (Escobar et al., 2012). Cells were seeded in 96-well microplates at 5×10^4 cells/well, and after monolayer formation they were tested with PSE or QE (31–500 μg/mL) for 24 h. Then, the medium was replaced with 200 μL of a 50 mg/mL solution of neutral red in EMEM. After 3 h of incubation at 37 °C, this medium containing dye was removed and the wells were washed twice with PBS. The dye was then extracted from the cells using acidified water/ethanol (1:49:50, v/v/v) mixture, after which, the cultures were agitated for 10 min and absorbance was measured at 540 nm.

Human PBMC cells (2×10^5) were cultured in 96-well microplates and incubated at 37 °C with 5% CO_2 and humidity for 18–24 h, before being exposed to increased concentrations of PSE or QE (31–250 μg/mL). Cell viability was evaluated by the TB dye exclusion assay (Mentel, Matthesb, Janta-Lipinski, & Wegner, 1996) using a Neubauer chamber for counting the viable cells, as described elsewhere (Cariddi et al., 2015). Our study was carried out at the Universidad Nacional de Río Cuarto and was approved by the Comité Institucional de Ética de la Investigación en Salud (CIEIS). In accordance with ethical standards, the healthy volunteers were fully informed about the study and signed an agreement authorizing the tests.

The cytotoxic effect in the three cell types was expressed as the percentage of cell proliferation with respect to control cells (0.05% DMSO; 100%). The data show the mean ± standard deviation (SD) of three wells per treatment and are representative of three experiments.

2.5. Cell morphology of the epithelial cell lines

The changes in the morphology of IEC-18 and Vero cells after incubation with PSE and QE (31–600 μg/mL) were analyzed by phase contrast using inverted (Eclipse TI-S, Nikon) and optical (Carl Zeiss, Jena, Germany) microscopes, which were both equipped with digital cameras. In addition, the maximum non-cytotoxic concentration (MNCC) was determined microscopically.

2.6. Interaction with ROS

2.6.1. Superoxide anion radical scavenging assay

Superoxide anion radical ($O_2^{\cdot -}$) scavenging activity was evaluated according to a procedure described by Vanden Braber et al. (2018). The assay used is based on $O_2^{\cdot -}$ generation by HAHC autoxidation, which reduces NBT to nitrite ($NBT^{+\cdot}$). In aqueous media, the organic radical-cation $NBT^{+\cdot}$ yields the stable cation monoformazan (MF^+), whose maximum absorption occurs at 560 nm. Briefly, different volumes of sample solution were mixed with 50 mM phosphate buffer (pH 8) containing 1 mM NBT, 1 mM EDTA, and 5 mM HAHC. After 1 h at 37 °C the absorbance at 560 nm was measured. The $O_2^{\cdot -}$ scavenging activity SA (%) was calculated as described below in Eq. (1), and the data were expressed as the effective concentration required to reach 50% of the radical scavenging (EC_{50}) value (mg/mL).

$$SA(\%) = (A_0 - A_x/A_0) \times 100 \quad (1)$$

where Ax is the absorbance in the presence of sample solution, and A_0 is the absorbance of the control.

2.6.2. Cytoprotection on MEN-treated IEC-18 cells

IEC-18 cells (5×10^4/mL) were seeded in 96-well plates and incubated in growing medium for 24 h, and MEN was used to induce oxidative stress (Marchionatti et al., 2009). Cells were treated with MEN at concentrations ranging from 5 to 50 μM for 24 h, and then 25 μM was selected (showing 50–60% of cell viability) to examine the cytoprotective effects of PSE and QE against MEN-induced oxidative stress. Two sets of experiments were performed. In the first one, cells were pretreated with 1.5, 3, 6 and 15 μg/mL of PSE or QE for 24 h and then exposed to MEN (25 μM) for 24 h (pre-treatment). In the second one, cells were incubated jointly with MEN (25 μM) and bioactive compounds (1.5, 3, 6 and 15 μg/mL) for 24 h (co-treatment). Similar methodologies have been previously reported by González-Sarrías et al. (2017). After each assay, cell viability was measured using the MTT assay described above.

2.6.3. Measurement of ROS by flow cytometry

IEC-18 cells were treated with PSE or QE (1.5 and 6 μg/mL) for 1 and 24 h to evaluate PSE and QE ROS production, and SOD was also added to analyze the mechanism of ROS generation. In other assays, cells were co-treated with QE or PSE (0.5 and 1.5 μg/mL) and MEN (1.5 μM) for 1 h, with the aim of examining the antioxidant effects of PSE and QE against MEN-induced oxidative stress. In both cases, cells were harvested by trypsinization and pelleted by centrifugation. The ROS were analyzed on a Becton Dickinson Accuri C6 Flow Cytometer in cell suspensions (1×10^6 cells) incubated with 1 μM in PBS for 15 min at 37 °C. In addition, the conversion of HE to ethidium by the superoxide anion was analyzed by flow cytometry on FL3 using a 620-nm band pass filter. Data were analyzed using FlowJo analysis software.

2.6.4. SOD activity

After co-treatments with QE or PSE (1.5 μg/mL) and MEN (1.5 μM) for 1 h of incubation, the IEC-18 cells were washed twice with PBS, resuspended and homogenized in lysis buffer (1 mM PMSF, 1 mM NaF, and 1% Triton X-100). The homogenate was cleared by centrifugation (10000 rpm) for 10 min at 4 °C, and the resulting supernatants were used for the enzyme assay. SOD activity was determined by reduction of NBT, measured spectrophotometrically at 560 nm as described previously (Liaudat et al., 2014). Results were expressed as SOD units/mg protein (one SOD unit is the enzyme activity that inhibits 50% the NBT reduction).

2.7. Statistical analysis

Data were analyzed by means of a one-way ANOVA and Bonferroni tests. Mean values were considered to be significantly different at $p < 0.05$. Statistical analyses were performed using GraphPad Prism 4 software (GraphPad Software, San Diego, CA, USA).

3. Results and discussion

3.1. Cytotoxicity assays

In order to evaluate the potential of PSE as a functional ingredient, the effect of PSE and QE on the viability of ileum epithelial cells (IEC-18) was investigated (Fig. 1A). Neither PSE nor QE showed any significant differences when tested at concentrations lower or equal to 300 μg/mL. In addition, at these concentrations, there were no differences observed with respect to the control treatment. However, at higher concentrations, both PSE and QE caused significant decreases in the percentage of cell viability, which was lowered by about 88 and 50% when concentrations of both PSE and QE were increased to 400 and 500 μg/mL, respectively, indicating a concentration-dependent effect. Thus, summing up, PSE revealed a similar activity (toxicity on IEC-18 cells) to that of QE, with neither causing significant cell viability loss if used at concentrations equal to or less than 300 μg/mL.

The cytotoxic effects of PSE on Vero cells (Fig. 1B) were also similar to those observed on IEC-18 cells. Concentrations equal to or lower than 300 μg/mL caused no significant differences to cell viability, with these values being similar to those of the QE and control treatments. In contrast, both PSE and QE at concentrations of 400 and 500 μg/mL showed similar effects, producing a significantly lower percentage of cell viability (about 80 and 60%, respectively, compared to the control treatment. At the highest concentration tested (500 μg/mL), the PSE toxicity on Vero cells was somewhat lower (higher cell viability percentage) than that observed for IEC-18 cells.

Human PBMC cells were used as a model to determine the cytotoxic

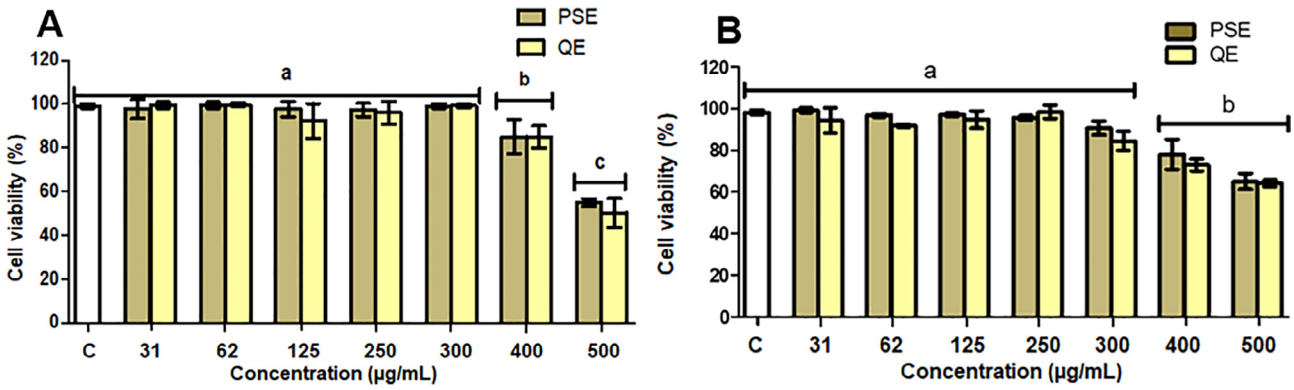

Fig. 1. (A) Cell viability, determined by the MTT assay, of IEC-18 cells treated with different concentrations (31–500 µg/mL) of PES and QE. (B) Viability, determined by the NRU assay, of Vero cells exposed to different concentrations (31–500 µg/mL) of PES and QE. Control cells with 0.05% DMSO, C. Values are presented as percentages (mean ± SD, n = 3 different experiments). Values with the same superscript letters are not significantly different from each other at $p < 0.05$.

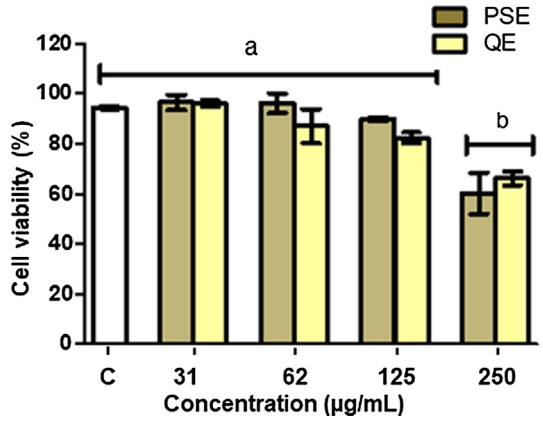

Fig. 2. Viability of human PBMC cells to different concentrations (31–250 µg/mL) of PES and QE, determined by the TB assay. Control cells with 0.05% DMSO, C. Values are presented as percentages (mean ± SD, n = 3 different experiments). Values with the same superscript letters are not significantly different from each other at $p < 0.05$.

effect and the potential risk to human health. Regarding the toxicity of PSE and QE on PBMC cells (Fig. 2), neither PSE nor QE were found to have any significant differences with the control treatment at concentrations of equal to or less than 125 µg/mL. In contrast, the highest concentration tested (250 µg/mL) decreased cell viability by levels close to 70%, with this concentration of PSE having a higher toxicity on human PBMC cells than that observed on either IEC-18 or Vero cells.

One general concern, based on many investigations, is that a compound is not considered to be cytotoxic, weakly cytotoxic or cytotoxic on cells, when the cell viability percentage is higher than 70, between 50% and 70% or less than 50%, respectively (Abdillahi, Verschaeve, Finnie, & Van Staden, 2012). Thus, according to these scientific findings and considering the cytotoxicity on both IEC-18 and Vero cell lines, the compounds present in PSE would not exert cytotoxic effects when the extract is used at concentrations equal or less than 300 µg/mL. However, cytotoxic effects on PBMC cells were observed at lower concentrations, and according to this assay, PSE revealed a weak cytotoxicity at 250 µg/mL. Taken together, these findings suggest that phenolic compounds from PSE have low cytotoxicity levels, which are comparable to those from QE.

Although the antioxidant properties of peanut skin phenolics are well-known, (Bodoira et al., 2017; Munekata et al., 2016; Yu, Ahmedna, & Goktepe, 2005), there is a paucity of research on their possible toxic effects. Consequently, information on this topic is important to be able to validate the potential use of agricultural residues such as sources of antioxidants or other nutraceutical compounds (Filippin et al., 2019). We previously evaluated the antioxidant activity of PSE as the scavenging activity of DPPH (2,2-diphenyl-1-picrylhydrazyl) radical, ABTS 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) cation radical and hydroxyl radicals (Bodoira et al., 2017), and determined that the IC_{50} value (extract concentration which causes a 50% decrease of the initial concentration of the corresponding radical) was between 3 and 12.5 µg/mL. In the present study, however, the concentration values at which the PSE showed a reduction in cell viability for IEC-18, Vero, and PBMC cells were between 20 and 100 times higher than those of the IC_{50} of free radical scavenging activity. Thus, our results show that PSE can exert a high antioxidant activity at a safe concentration range for normal cells.

A pioneering study in humans by Egert et al. (2008) reported a low oral bioavailability of QE, which ranged from 76.1 µmol min/L (22.8 µg min/mL) to 305.8 µmol min/L (91.8 µg min/mL) when volunteers were administrated 50, or 150 mg/day of this compound for two weeks. Also, median maximum plasma concentrations of QE 431 nmol/L (0.13 µg/mL) were observed 360 min after intake of 150 mg QE, which were much lower than the weak cytotoxicity concentration observed against PBMC cells, thereby demonstrating that PSE does not present a risk to human health.

Lewis, Harris, Sanders, White, and Dean (2013) evaluated the antioxidant and anti-inflammatory activity of peanut skin extract containing high levels of procyanidins and other phenolic compounds. Its anti-inflammatory effects in the murine monocyte/macrophage cell line (RAW 264.7) were evaluated upon induction with the inflammatory marker lipopolysacharride. Peanut skin extract caused a small decrease in RAW 264.7 cell viability (around 70% for a concentration of 50 mg/mL). In addition, peanut skin extract pretreatment of RAW 264.7 cells induced with LPS exerted an anti-inflammatory effect with inhibition of the expression of the pro-inflammatory enzyme cyclooxygenase-2, and a reduction in the levels of prostaglandin E2 and nitrous oxide.

Recently, Filippin et al. (2019) reported that peanut leaf extracts, composed mostly of polyphenols including phenolic acids, flavonoids and tannins, did not cause any significant effects on PBMC cell viability. Other studies have been carried out on the toxicity of phenolic compounds that might be present in the peanut skin. Of these, QE, a polyphenol widely present in many plant foods, is one of the most studied. Related to this, Bharathi et al. (2017) found no toxicity of QE on normal cells (PBMC and IEC-6 cells) at a concentration of 80 µM (24 µg/mL), but it selectively induced apoptosis in cancer cells. In agreement with these findings, other investigations have indicated that QE has a greater activity on tumor cells than on normal cells (Du et al., 2010; Park & Min, 2011).

The cytotoxicity of polyphenols has been widely investigated in normal cells (Atsuo, Asaki, Aga, & Aneko, 2005; González-Sarrías et al., 2017; Smeriglio et al., 2019) and cancer cell lines (Aherne & O'Brien

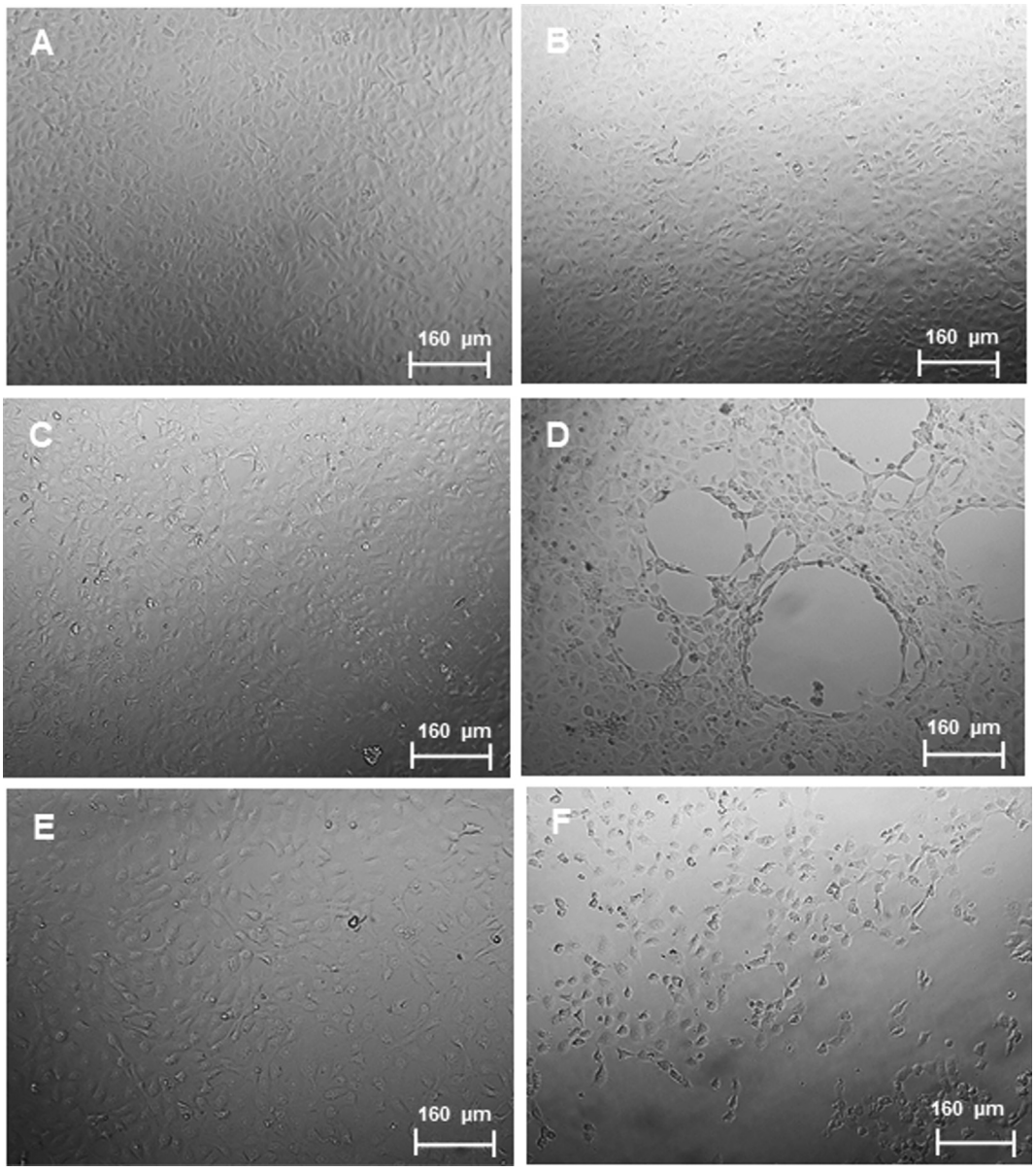

Fig. 3. Microscopic images (×10) of IEC-18 cells, obtained both before and after treatment with PSE and QE on cells. (A) Untreated cells and (B) cells treated with DMSO 0.05% were used as controls. (C) MNCCs of PSE (300 µg/mL), (D) cells treated with 400 µg/mL of PSE, (E) MNCCs of QE (250 µg/mL) and (F) cells treated with 300 µg/mL of QE.

Nora, 2000; Carolina et al., 2013). It is well-known that flavonoids can act either as pro-oxidants or as antioxidants, depending on the flavonoid concentration and/or cell type (Atsuo et al., 2005). Atsuo et al. (2005) have demonstrated that flavones and flavonoids, especially QE, exert important cytotoxicity activity against human cells. In that study, cytotoxicity was evaluated on lung embryonic fibroblasts (TIG-1) and umbilical vein endothelial (HUVE) cells, with it being concluded that their cytotoxicity may be intimately related to both their incorporation efficiency and intracellular ROS-generating ability. In another investigation, the cytotoxicity of procyanidin fractions from grape pomace and pine bark was evaluated against the human keratinocyte normal cell line HaCaT and mouse fibroblast normal cell line 3T3 (Ugartondo et al., 2007). It was observed that the fractions varied in their mean molecular weights, the degree of polymerization (monomeric cathechins, oligomeric catechins procyanidins), and the percentage of galloylation (presence of gallate esters) of its components, with all these fractions revealing a certain degree of toxicity. These authors reported a strong correlation between the antioxidant and cytotoxic activities for all fractions, with the best antioxidant fraction being the most toxic one to cells. Moreover, the cytotoxic concentrations were approximately 6 times higher than the concentrations with high antioxidant activity.

3.2. Cell morphology of epithelial cell lines

The cell morphology was observed and evaluated microscopically for any possible changes, with the MNCC being taken as the maximum concentration tested which did not show any detectable cytotoxic effects microscopically (Ramadan, Shawkey, Rabeh, & Abdellatif, 2019). The MNCCs determined in IEC-18 cells were 300 and 250 µg/mL for PSE and QE, respectively (Fig. 3). In Fig. 4, it can be observed that the MNCCs evaluated in Vero cells were 300 µg/mL for PSE and QE. The number of cells from both cell lines decreased at these concentrations, which is consistent with the results obtained in the cytotoxicity assays, where concentrations of 400 µg/mL of both bioactive compounds slightly decreased the viability of the IEC-18 and Vero cells.

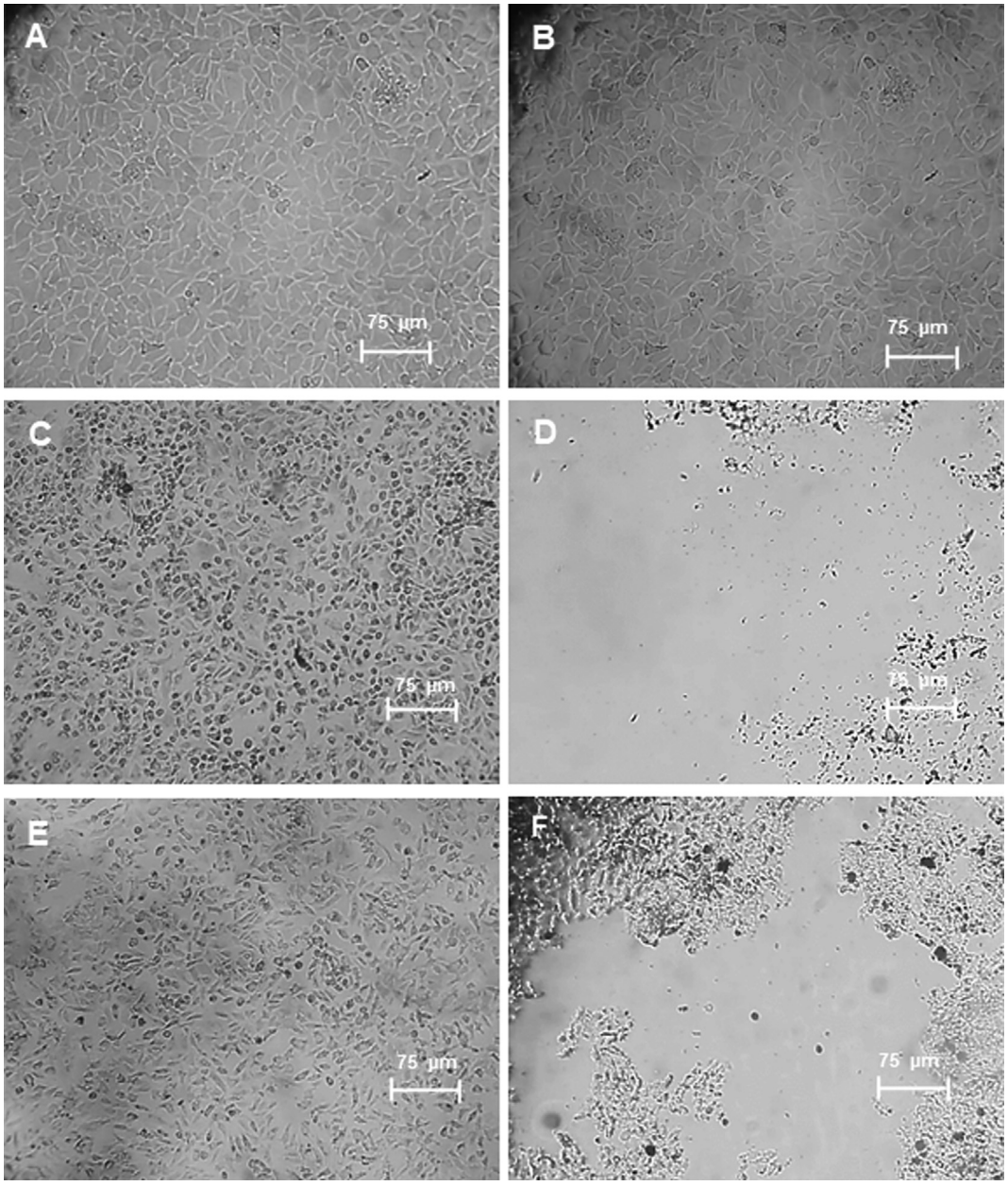

Fig. 4. Microscopic images (×10) of Vero cells both before and after treatment with PSE and QE. (A) Untreated cells and (B) cells treated with DMSO 0.05% were used as controls. (C) Cells treated with 300 µg/mL (MNCCs) and (D) cells treated with 400 µg/mL of PSE, (E) cells treated with 300 µg/mL of QE (MNCCs) and (F) 400 µg/mL of QE.

3.3. Interaction with ROS

3.3.1. $O_2^{\cdot -}$ radical scavenging activity

The $O_2^{\cdot -}$ radical scavenging activity of PSE and QE was measured and the EC_{50} value (mg/mL) was calculated from a linear regression fit of $O_2^{\cdot -}$ SA (%) *versus* concentration (Fig. S1 of the supplementary material). The EC_{50} values obtained for QE and PSE were 0.018 and 0.13 mg/mL, respectively, showing that QE is approximately 7 times more reactive against the $O_2^{\cdot -}$ radical. This EC_{50} value obtained for QE is consistent with that previously reported by Chun, Kim, and Lee (2003). The lower scavenging activity shown by PSE may be due to the previously demonstrated pro-oxidant action (hydroxyl radical and $O_2^{\cdot -}$ radical formation) of the catechin, epicatechin and procyanidin dimers (Azam, Hadi, Khan, & Hadi, 2004), already identified as being the major components of the extract (Bodoira et al., 2017).

3.3.2. Cytoprotective effect against oxidative stress

The cytoprotective effect of PSE against the oxidative stress generated by MEN on IEC-18 cells was evaluated. Pre-treatments using PSE and QE at concentrations between 1.5 and 15 µg/mL significantly decreased MEN-induced cytotoxicity on IEC-18 cells, with the mean percentages of cytotoxicity depletion found to be 50% (PSE) and 40% (QE), and with no significant differences observed with the control without stressor (Fig. 5). The co-treatment assays revealed fewer cytoprotective effects than those from pre-treatment ones. Nevertheless, co-treatment with PSE or QE was found to increase significantly the viability of cells (50% with MEN compared to 80% after co-treatment with PSE or QE).

The aforementioned results are in general agreement with those of Aherne and O'Brien Nora (2000) who reported that a 50 µM (15 µg/mL) concentration of QE may protect MEN-induced DNA damage in Caco-2 cells. MEN is a quinone that can be toxic by causing oxidative stress and by interaction with thiol groups of essential molecules, such as proteins

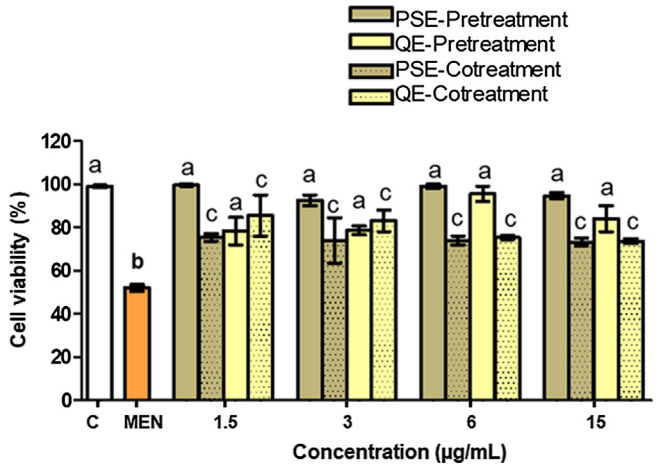

Fig. 5. Cytoprotective effects of pre- and co-treatment of PSE and QE (1.5–15 µg/mL) against MEN (25 µM). Control cells with 0.05% DMSO, C. Values are presented as percentages (mean ± SD, n = 3 different experiments). Values with the same superscript letters are not significantly different from each other at $p < 0.05$.

and glutathione. In the former case, the oxidation of semiquinone radicals by molecular oxygen results in the production of $O_2^{\cdot -}$, generating H_2O_2, which is involved in the induction of DNA damage by acting as a precursor of free radicals (Aherne & O'Brien Nora, 2000; Boots et al., 2007; Marchionatti et al., 2009). Thus, the cytoprotective effects of the PSE used in the present study, which is composed of many phenolic compounds that have potent scavenging activities against varied free radicals such as peroxyl, hydroxyl and $O_2^{\cdot -}$ (Bodoira et al., 2017; Larrauri et al., 2016), might be mainly attributed to its antioxidant (antiradical) properties. In support of this hypothesis, a phenolic extract from peanut leaves (PEPL), which has some compounds in common with PSE, was found to ameliorate the damaging effects of hydrogen peroxide (H_2O_2) on human PBMCs (Filippin et al., 2019). In agreement with these findings, QE increased C3A cell viability when exposed to the pro-oxidant 2,2′-azobis(2-amidinopropane hydrochloride) (Joko et al., 2017), and also had protective effects on H_2O_2-induced DNA damage (Boots et al., 2007), with these effects being attributed to its scavenging activity towards peroxyl radicals.

Guo et al. (2007) studied the possible cytoprotective effects of oligomeric and polymeric procyanidin fractions isolated from grape seeds on ethanol-induced DNA damage in mouse brain cells. These authors found that the oral administration of grape seed oligomer and polymer procyanidins (25, 50, and 100 mg/kg animal) for 3 days as a pre-treatment or in a co-treatment with ethanol for 30 days could significantly inhibit DNA damage induced by ethanol in brain cells. Catechins, the monomeric units of procyanidins, have also demonstrated protective effects against DNA damage by free-radical scavenging activities and the quenching of singlet oxygen (Mukai, Nagai, & Ohara, 2005).

These above results indicate that the cytoprotective effect exerted by PSE could be due to the antioxidant action of its major components procyanidin dimers, catechin and QE.

3.3.3. ROS generation

The QE and PSE ROS production was analyzed in QE and PSE treated-IEC-18 cells by flow cytometry. The potential mechanism of the protective effects of flavonoids against oxidative stress is based on their capacity to decrease the ROS levels. However, antioxidants can actually have pro-oxidant activity under certain conditions, thus diets with large amounts of a single antioxidant may be harmful to the health (Shahidi & Ambigaipalan, 2015). In our study, we found that 24 h of incubation with QE (6 µg/mL) and PSE (1.5 µg/mL) significantly increased ROS formation in IEC-18 cells, compared with control. It was also observed that the superoxide anion was involved in this process, with ROS production decreasing when SOD was added (Fig. 6). However, no significant changes between QE and PSE treated and control IEC-18 cells were observed when cells were exposed to a concentration of 1.5 µg/mL for times less than 24 h (Fig. 6). Moreover, to further evaluate their antioxidant potential, the ROS production of QE and PSE was tested against the oxidative stress generated by MEN (1.5 µM) on IEC-18 cells. These results showed that both compounds (0.5 and 1.5 µg/mL) significantly inhibited ROS production in IEC-18 cells ($p < 0.05$ vs. MEN; Fig. 7). In addition, SOD activity was evaluated in QE and PSE (1.5 µg/mL) treated- IEC-18 cells, which revealed that for the QE and PSE concentrations used, there was no increased SOD activity in the IEC-18 cells ($p < 0.05$ vs. C; Fig. S2 of the supplementary material). SOD activity was also evaluated against MEN (1.5 µM), and it was found that QE and PSE (1.5 µg/mL) reduced SOD activity in the IEC-18 cells ($p < 0.05$ vs. MEN; Fig. S2 of the supplementary material).

It is well known that polyphenols of peanut skin can protect against an excessive ROS being produced in oxidative stress (Costa De Camargo

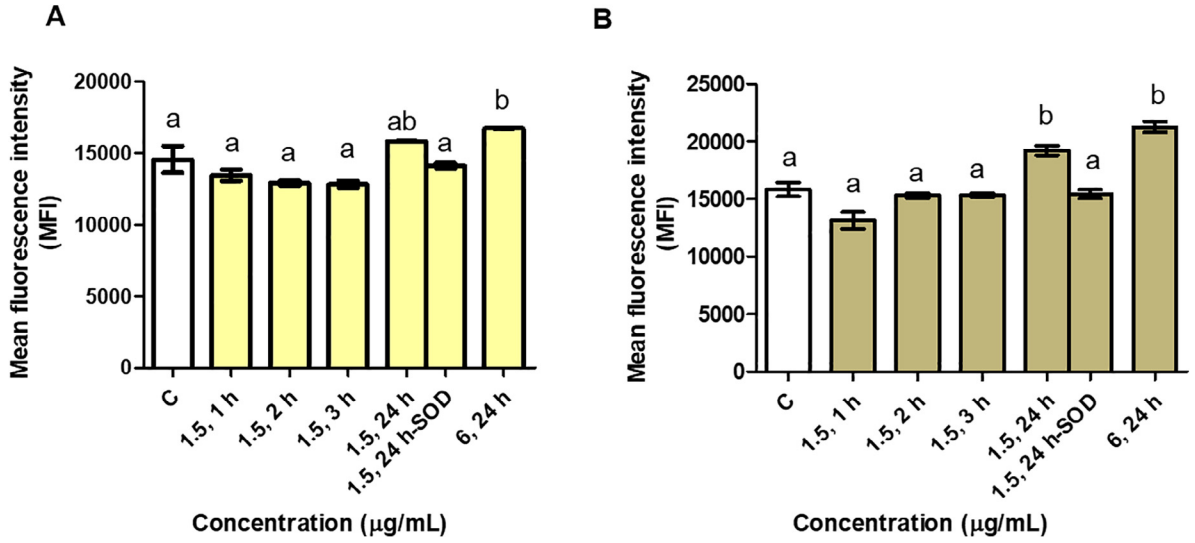

Fig. 6. Production of ROS in IEC-18 cells analyzed by flow cytometry and production of ROS at different concentrations of QE (A) and PSE (B), and at different times of incubation (1, 2, 3 and 24 h). Values are presented as mean fluorescence intensity (MIF) (mean ± SD, n = 3 different experiments). Values with the same superscript letters are not significantly different from each other at $p < 0.05$.

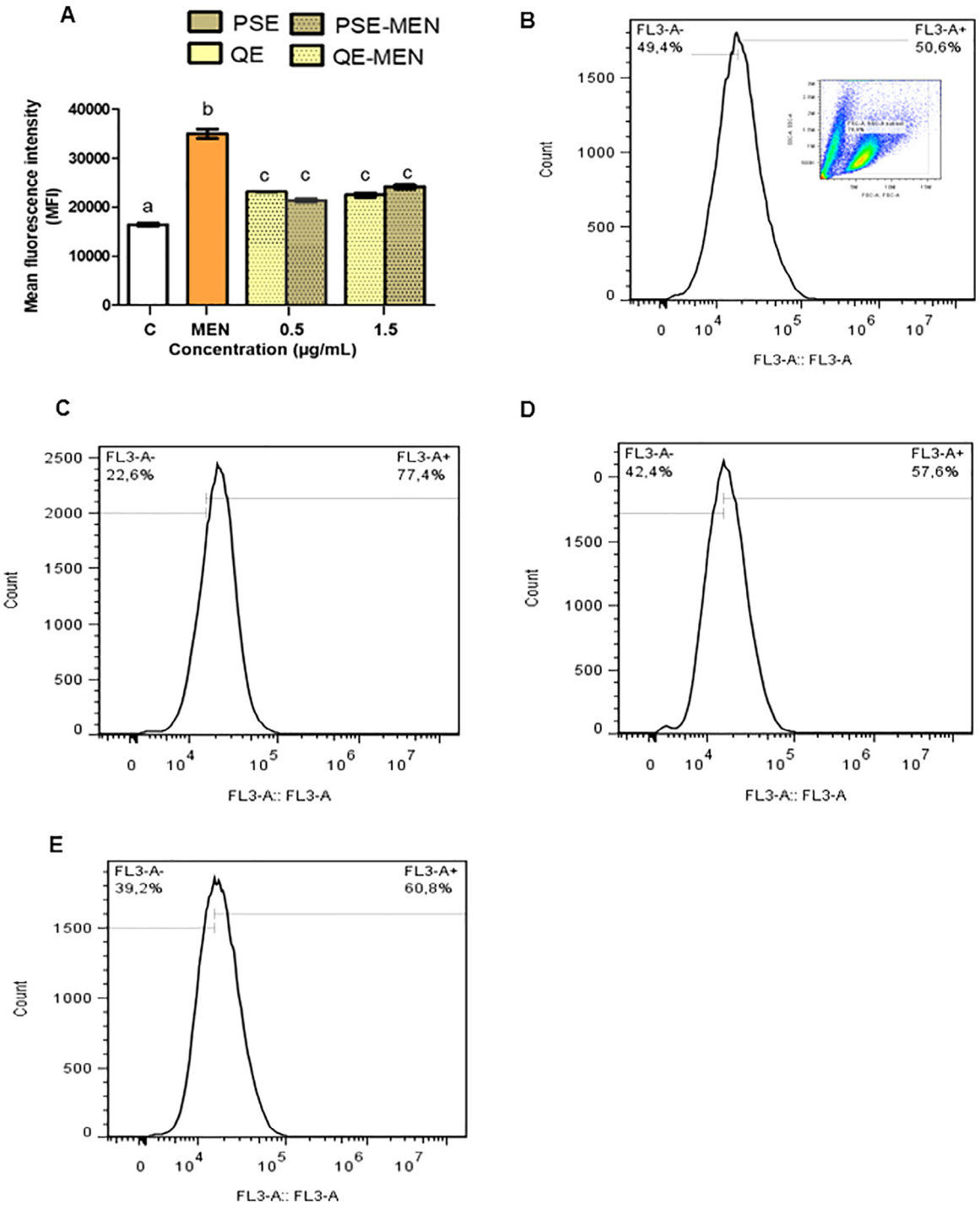

Fig. 7. Effect of co-treatment of PSE and QE (0.5 and 1.5 µg/mL) on ROS formation against MEN (1.5 µM) in IEC-18 cells analyzed by flow cytometry. (A) Values of mean fluorescence intensity (MIF) (mean ± SD, n = 3 different experiments). Values with the same superscript letters are not significantly different from each other at $p < 0.05$. (B) Control cells with 0.05% DMSO, (C) MEN 1.5 µM, (D) MEN 1.5 µM and QE 1.5 µg/mL, (E) MEN 1.5 µM and PSE 1.5 µg/mL.

et al., 2017). In our investigation, we observed pro-oxidant and anti-oxidant effects of polyphenols in IEC-18 cells, showing that these effects depended on the concentration and incubation time of these compounds. QE and PSE, when incubated for 24 h at concentrations of 6 µg/mL and 1.5 µg/mL, respectively, caused increased levels of ROS in IEC-18 cells. Our results are in line with previous studies which show that QE and the polyphenols present in PSE (catechins) play paradoxical roles, inducing pro-oxidant and cytotoxic effects under appropriate conditions due to the formation of ROS (Azam et al., 2004; Jo, Rietjens, & Narimantas, 1999) and of intermediate metabolites (Silvia et al., 2012). It has been postulated that the pro-oxidant action of polyphenols may be an important mechanism of their anticancer properties (Azam et al., 2004; Hadi, Asad, Singh, & Ahmad, 2000), with Azam et al. (2004) showing that the production of the superoxide radical increased with incubation time. Previous research has proposed that mobilization of endogenous copper, an important metal ion present in chromatin and closely associated with DNA bases (in particular, guanine), is a consequent pro-oxidant action (Hadi et al., 2000). Other authors (Bharathi et al., 2017) have reported that levels of ROS production were significantly increased in 40 mM (12 µg/mL) QE treated

HT29 cells, although significant changes between QE treated and control IEC-6 cells were not observed (the time of incubation of QE was not reported in this work). This is in line with several reports that indicate that the serum and tissue concentrations of copper are greatly increased in various malignancies (Hadi et al., 2000).

Our study, on the other hand, showed that QE and PSE gave bioprotection against stressor cells (MEN) at concentrations of 0.5 and 1.5 μg/mL (1 h of incubation). Both these compounds also inhibited ROS production in the stressed IEC-18 cells, which is consistent with a previous investigation (Alía et al., 2006). These authors observed that ROS generation induced by *tert*-butyl hydroperoxide was significantly reduced when HepG2 cells were pretreated for 2 or 20 h with 10 μM (3 μg/mL) and for 20 h with 5 μM (1.5 μg/mL) QE. Moreover, other authors have reported exposure of PBMCs to PEPL at 1 μg/mL being able to reduce reactive species production (Filippin et al., 2019). In agreement, our results obtained for SOD activity show that QE and PSE prevented an increase in enzyme activity (at 1.5 μg/mL with 1 h of incubation) by MEN in IEC-18 cells. Similar effects were also observed in HepG2 cells treated with QE (Alía et al., 2006).

4. Conclusions

The peanut skin phenolic extract, whose composition we had previously obtained using GRAS solvents (water and ethanol), has a vast array of phenolic compounds (catechin, epicatechin, procyanidin and proantocyanidin dimers, and QE) with important biological properties and potent antioxidant capacities. Our results of the present study indicate that the peanut skin phenolic extract does not present cytotoxicity against normal cells in the concentration ranges with maximum free radical scavenger activity, and that it exerts a bioprotective effect at a safe concentration range for normal cells. This suggests that peanut skin phenolic extract can be safely applied in new antioxidant bioactive ingredients in the formulation of functional foods. Overall, the findings from this study have provided useful information for the characterization of the biochemical and biological properties of polyphenols. Further studies, involving cytotoxicity analyses with *in vivo* models, are now necessary to obtain a better understanding of the molecular mechanisms underlying the protective effects of peanut polyphenols.

5. Ethics statements

The research did not include animal experiments.

Studies on human PBMCs cells was carried out at the Universidad Nacional de Río Cuarto and was approved by the Comité Institucional de Ética de la Investigación en Salud (CIEIS). In accordance with ethical standards, the healthy volunteers were properly informed of the study and signed an agreement authorizing the test.

CRediT authorship contribution statement

Yanina E. Rossi: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing - original draft, Funding acquisition. **Luciana P. Bohl:** Conceptualization, Methodology, Validation, Formal analysis, Investigation. **Noelia L. Vanden Braber:** Methodology, Investigation. **María B. Ballatore:** Methodology, Validation, Investigation. **Franco M. Escobar:** Methodology, Validation, Resources, Investigation. **Romina Bodoira:** Resources, Investigation. **Damián M. Maestri:** Conceptualization, Resources, Funding acquisition. **Carina Porporatto:** Conceptualization, Funding acquisition. **Lilia R. Cavaglieri:** Conceptualization, Funding acquisition. **Mariana A. Montenegro:** Conceptualization, Resources, Supervision, Project administration, Funding acquisition, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jff.2020.103862.

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