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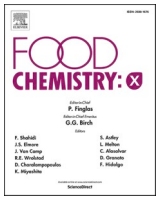

Elderberry (*Sambucus nigra* L.) extracts promote anti-inflammatory and cellular antioxidant activity

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ABSTRACT

Despite the high value of Portuguese elderberries, recognized for decades by European markets, only a few studies address their beneficial effects at cellular level. Aiming to explore the anti-inflammatory and the cellular antioxidant potential characterized extracts from the three main Portuguese elderberry cultivars (Sabugueiro, Sabugueira, Bastardeira) were used. Lipopolysaccharide-stimulated RAW 264.7 cells pre-exposed to elderberry extracts exhibited dose-dependent inhibition of nitric oxide release, evidencing anti-inflammatory activity. Concerning cellular antioxidant protection, HepG2 and Caco-2 cells pre-exposure to elderberry extracts (50 μg/mL) prevented up-to 90 % of *tert*-butyl hydroperoxide (*t*-BOOH)-induced toxicity. In Caco-2 cells, elderberry extracts prevented glutathione depletion, reactive oxygen species production, abnormal morphological changes and DNA fragmentation, in response to *t*-BOOH oxidative insult. Results demonstrated that elderberries have high potential in reducing cellular oxidative stress as well as in preventing inflammatory processes. Thus, elderberries have high potential as health promoters, acting as functional foods or as sources of nutraceuticals.

Introduction

Inflammation is a complex and natural defence mechanism in response to harmful stimuli, modulated by the immune system, involving monocytes and macrophages activation (Jiang et al., 2020). Following macrophages activation, several inflammatory cytokines and/or mediators are released, namely interleukins, tumour necrosis factor alpha (TNF-α), prostaglandin E_2 (PGE_2), reactive oxygen species (ROS) and nitric oxide (NO) (Ho, Wangensteen, & Barsett, 2017). NO can be synthesized by nitric oxide synthases (NOS; EC 1.14.13.39), a family of enzymes comprising neural (nNOS), endothelial (eNOS) and inducible (iNOS) isoenzymes. After an inflammatory stimulus, iNOS is rapidly synthetized and induced, playing a pivotal role in the defence mechanism (Ho et al., 2017). However, elevated concentrations of NO or persistent inflammation status lead to cellular damage and to chronic inflammatory diseases (Pergola, Rossi, Dugo, Cuzzocrea, & Sautebin, 2006), as cardiovascular diseases, rheumatoid arthritis, among others (Ho et al., 2017). Thus, controlling NO levels, either through its sequestration or by decreasing its production, represents a preventive and crucial strategy against inflammatory diseases; and, natural compounds, such as polyphenols, polysaccharides, among others, present anti-inflammatory activity (Tzianabos, 2000). ROS play a pivotal role as signalling molecules, namely in inflammatory processes, but also result from other physiological mechanisms, being the oxidative phosphorylation the main source of cellular superoxide anion. However, high levels of ROS lead to cascades of events involving the oxidation of lipids, proteins, DNA and other constitutive molecules (Doktorovová et al., 2014). The association between oxidative stress and chronic diseases, such as neurodegenerative and cardiovascular diseases or cancer has been reported (Alía, Ramos, Mateos, Bravo, & Goya, 2005; Alía et al.,

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2006; Fernández-Tomé et al., 2014). Among the different ROS, peroxides can be decomposed to peroxyl radicals (ROO•) accelerating lipid peroxidation, DNA damage, and thus causing cell damage (Alía et al., 2005; Doktorovová et al., 2014). Endogenous and exogenous antioxidant defences play a crucial role counteracting the increase in oxidative stress. As exogenous molecules, vitamins (C and E) and polyphenols act as antioxidants, a property that arise from their molecular structure. For example, the hydroxyl groups present on phenolic structure are hydrogen donors that can react with reactive ROS breaking the cascades and the generation of new radicals (Alía et al., 2005; Souto et al., 2019). Therefore, it is necessary to find new food sources with components that promote anti-inflammatory and antioxidant action at the cellular level.

Sambucus nigra L. fruit (elderberry) and flower (elderflower) have been used to treat colds, flu and inflammatory-related conditions (Ferreira, Silva, & Nunes, 2020a). The anti-inflammatory role of elderflowers was already demonstrated in different studies (Harokopakis, Albzreh, Haase, Scannapieco, & Hajishengallis, 2006; Ho et al., 2017; Ho, Zou, Aslaksen, Wangensteen, & Barsett, 2016), however, few studies are available concerning the anti-inflammatory role of elderberries using *in vitro* cellular models. Ho et al. (2017) reported that elderberry extracts (elderberries from Norway) inhibited NO release from lipopolysaccharide (LPS)-activated RAW 264.7 cells; and Olejnik et al. (2015) reported anti-inflammatory effect of *in vitro* digested elderberry products (fruits from Poland), by reducing interleukin-6 (IL-6), TNF-α and PGE2 release from LPS-stimulated RAW 264.7 cells. Elderberry extracts also modulated the levels of inflammatory cytokines in diabetic rats (Badescu, Badulescu, Badescu, & Ciocoiu, 2015). Moreover, elderberries have a high content in polyphenols, e.g., phenolic acids, flavonols, proanthocyanidins and anthocyanins, that are associated with a beneficial antioxidant effect, although most of this knowledge comes from results obtained by conventional colorimetric quantitative methods, such as the scavenging of ABTS radical or of other radicals (Ferreira et al., 2020a; Sidor & Gramza-Michałowska, 2015). Therefore, there is a need to obtain evidence of this effect using cellular models.

As Portuguese elderberries have been recognized by the different European markets and industries for their high quality but few studies are available concerning their components' biological activities, the main aim of this work was to evaluate the potential health benefits of the three main Portuguese elderberry cultivars ('Sabugueiro', 'Sabugueira' and 'Bastardeira'), from Varosa Valley, by assessing the anti-inflammatory and the cellular antioxidant effect of elderberry extracts. anti-inflammatory effect will be assessed by the extracts' ability to reduce NO release from LPS-stimulated RAW 264.7 cells, and the antioxidant cellular protection by the ability to prevent the oxidative stress injuries promoted by exposure of Caco-2 and HepG2 cells to *tert*-butylhydroperoxide (*t*-BOOH). These two cell lines [Caco-2 (enterocyte) and HepG2 (hepatocyte) cell models], due to their characteristics, are usually applied as reliable models in biochemical and nutritional studies, as physiologically they are exposed to high levels of ROS. So far as we known, this work reports the first approach to evaluate the beneficial effect of elderberries against the deleterious injuries promoted by a potent oxidant, *t*-BOOH, in Caco-2 and HepG2 cells, highlighting the health promoting effect of these berries.

Materials & methods

Elderberries: Sampling and extraction

In this study, fruits were harvested from three elderberry cultivars, 'Sabugueiro', 'Sabugueira' and 'Bastardeira', cultivated in the same field located in Varosa Valley, Moimenta da Beira, Portugal. The samples were collected on the optimum maturation stage (end of August), from four different points of the shrub and from 5 different shrubs, for each cultivar, early in the morning and conditioned in refrigerated boxes for transportation. All shrubs were exposed to the same factors (e.g. sunlight, irrigation, absence of pesticides, etc.). Extraction methods and chemical composition analysis of extracts was previously described in Ferreira, Silva, Silva, and Nunes (2020b). Briefly, freeze-dried elderberries were exhaustively extracted (8 times) with acidified methanol (1 % HCl). Elderberry extract (E) of each elderberry cultivar was further fractioned by SPE (solid-phase extraction), using C18 cartridges, where methanol elution allowed to obtain a rich-phenolic extract (M), and water elution allowed to obtain a non-phenolic extract rich in polar compounds (H) (Ferreira et al., 2020b). The extracts and fractions were lyophilized and kept in a dry place and protected from light until being used.

Cell cultures and their maintenance

Caco-2 (human colon adenocarcinoma, CLS, Eppelheim, Germany), HepG2 (human hepatoma cell line; ATCC, Rockville, MD, USA) and RAW 264.7 (mouse macrophages, Abelson murine leukemia virus-induced tumor, CLS, Germany) cells were maintained in DMEM (Dulbecco's Modified Eagle Medium) containing 25 mM glucose supplemented with 10 % (v/v) fetal bovine serum (FBS; Gibco, Life technologies), 2 mM L-glutamine (Gibco, Life technologies), 100 U/mL penicillin (Life technologies) and 100 μg/mL streptomycin (Life technologies) at 37 °C in a normal atmosphere of 5 % CO_2 in air and were handled as described (Silva et al., 2020).

Cell viability/cytotoxicity assay

In order to select the range of non-cytotoxic concentrations of elderberry extracts [total elderberry extract (E), rich-phenolic extract (M) and non-phenolic extract (aqueous fraction extract (H)], the cytotoxicity of each extract was first evaluated in Caco-2, HepG2 and RAW 264.7 cells. Cells were grown to near confluence, then were subjected to trypsin action (Caco-2 and HepG2) or were scratched off from the flasks (RAW 264.7). After being detached from the culture flask, suspended cells in fresh culture medium were counted (TC10^{TM}, automated cell counter, BIORAD, Portugal), then diluted in culture media, at density of $5x10^4$ cells/mL (Silva et al., 2020). Cells were then seeded onto 96-well microplates (at $5x10^4$ cells/well) and were maintained in the CO_2 incubator, to adhere and stabilize for 24 h. Then, culture media was removed and cells were exposed to elderberry extract solutions at different concentrations (Caco-2 and HepG2: up to 750 μg/mL; RAW 264.7: up to 1 mg/mL), made by dilution in FBS-free culture medium, for 24 h or 48 h. After the exposure time, cell viability was evaluated by Alamar Blue® assay (Invitrogen, Alfagene, Portugal), for that the incubation media was removed and immediately replaced by Alamar Blue solution (10 % (v/v), in FBS-free culture medium; 100 μL/well). Absorbance was read, after 5 h incubation, at 570 nm (reduced; resorufin) and 620 nm (oxidized; resazurin), using a microplate reader (Multiskan EX; MTX Lab Systems, Inc., Bradenton, FL, USA), since metabolic activity results in dye conversion from its oxidized to reduced form, which is accompanied by a colour change, thus the percentage of AB reduction is proportional to cell viability. The percentage of AB reduction was calculated according to the equations recommended by the manufactures, and as described in Andreani et al. (2014). In each assay, controls were made, consisting of non-treated cells (positive control) and of Alamar Blue solution alone (negative control). Cell viability results are expressed as % of positive control (n = 3 different experiments, each one in quadruplicates), for details see Silva et al. (2020).

Anti-inflammatory activity assessment

Anti-inflammatory activity was evaluated using LPS-stimulated RAW 264.7 macrophage cells as described by Martins-Gomes et al. (2018) with some modifications. Briefly, RAW 264.7 cells, at $5x10^4$ cells/mL in complete culture medium, were seeded in 96-well plates (100 μL/well) and were let to adhere and stabilize for 48 h in the incubator. As the

extracts are pink in colour, to avoid interference with absorbance readings, pre-exposure to the extracts was made before challenging the cells with LPS. Thus, after stabilizing, cells were exposed to the different elderberry extracts, at 10, 25, 50 and 100 μg/mL, for 24 h. After this incubation time, the extract solutions were removed, cells were washed with PBS (phosphate-buffered saline) and then were incubated, for additional 24 h, with FBS-free culture media without (negative control) or with 1 μg/mL LPS, to induce NO production and release to the supernatant. NO rapidly decomposes into NO_2 (nitrite). Then, 50 μL of each supernatant was transferred to a new 96-wellplate and then 50 μL of Griess reagent [(0.1 % (w/v) *N*-(1-naphtlyl) ethylenediamine dihydrochloride in water and 1 % (w/v) sulphanilamide in 5 % H_3PO_4 (v/v)] was added to each well, incubated for 10 min in the dark and then the absorbance was measured at 550 nm (Multiskan EX microplate reader, MTX Labsystems, USA). Quantification of nitrite production was made using a calibration curve of sodium nitrite (0 – 100 μM), as described in Silva et al. (2020). Results are expressed as percentage of positive control (cells only exposed to LPS), as mean ± S.D., of 3 independent experiments (each one done in quadruplicates).

Assessment of potential protection of elderberry extracts against tert-butyl hydroperoxide (t-BOOH)

To evaluate the elderberry extracts' protective effect against the oxidative effect of *t*-BOOH, crude elderberry extract (E) and rich-phenolic extract (M) were selected. Assays were performed with a single dose (50 μg/mL) of all extracts and in a dose–response way using different concentrations (up to 200 μg/mL) of Bastardeira extracts, as representative extract. Thus, Caco-2 and HepG2 cells were pre-treated with elderberry extracts (E and M), for 4 h, then the medium was discarded and replaced with fresh medium, for 16 h. Then, cells were challenged with 250 μM of *t*-BOOH, diluted in FBS-free culture medium, for 4 h (Carvalho, Franklin, Dias, & Lima, 2014). Finally, cell viability was evaluated using the Alamar Blue assay as described above, results are expressed as % of positive control (n = 3 different experiments, each one in quadruplicates) as mean ± S.D.

Bright field and fluorescence microscopy assay to assess changes in cell morphology, DNA integrity and mitochondrial potential

In order to observe the morphological changes in Caco-2 cells promoted by exposure to *t*-BOOH as well as the effect of exposure to elderberry extracts; Caco-2 cells were pre-treated with 50 μg/mL of elderberry extracts (elderberry extract (E) and rich-phenolic extract (M)), for 4 h. Then, the medium was discarded and replaced with fresh medium for 16 h. After that, cells were exposed to 100 or 250 μM of *t*-BOOH for 4 h. Then, to assess the DNA integrity and the mitochondrial depolarization, cells were incubated with Hoechst 33,342 probe (5 μg/mL) (Martins-Gomes, Souto, Cosme, Nunes, & Silva, 2019) and with JC-1 (5 μg/mL), respectively (both dyes from Invitrogen Life-Technologies, Porto, Portugal). After incubation, cells were observed under fluorescent microscope (Olympus IX51) coupled with a CCD camera, and photographs were acquired using Cell A^ image acquisition software.

Flow cytometry to assess oxidative stress markers

To determine oxidative stress caused by cell exposure to *t*-BOOH, as well as to assess the protective potential of elderberry extracts, we measured the levels of intracellular glutathione (GSH), cellular lipid peroxidation (LP) and intracellular reactive oxygen species (ROS), by using one-color and two-color flow cytometry (BD Accuri™ C6 cytometer, Becton Dickinson, Ca, USA) and 5000 gated events were collected from each sample, as described by Domínguez-Perles, Guedes, Queiroz, Silva, and Barros (2016) with some modifications. Briefly, Caco-2 cells were seeded in 12-well microplates ($5x10^4$ cells/mL), then cells were treated for 4 h with 50 μg/mL of each elderberry extracts (elderberry extract (E) and rich-phenolic extract (M)), after incubation the medium was discarded and replaced with fresh medium for 16 h. Then the cells were exposed to 100 or 250 μM of *t*-BOOH for additional 4 h. After exposure to *t*-BOOH, cells were washed with PBS, detached with trypsin, and centrifuged for 5 min at 3000 rpm (bench micro-centrifuge). The supernatants were removed and cells were resuspended in PBS and divided in three sets for the following analysis by flow cytometry: 1) To assess GSH levels, cells were incubated with 40 μM Mercury Orange ([1-(4-chloromercurphenyl-azo-2-naphtol); Sigma-Aldrich (Merck, Germany)] for 5 min, at room temperature, in the dark. 2) To assess the lipid peroxidation, cells were incubated with 10 μM DHPE-FITC (Invitrogen, Life-Technologies, Porto, Portugal) for 20 min, at room temperature in the dark. 3) To assess intracellular ROS, cells were incubated with 10 μM DCFDA (2′,7′-dichlorofluorescein diacetate; Invitrogen, Life-Technologies, Porto, Portugal), diluted in FBS-free culture medium, for 45 min, at 37 °C, in the dark. After incubation, 5 μL of propidium iodide (PI, at 50 μg/mL) was added to each sample before the flow cytometry analysis.

Flow cytometry analysis was performed using BD Accuri™ C6 Software, version 1.0.264.21 (Becton Dickinson, CA, USA), from at least 3 different experiments (n = 3; each one in duplicates).

Statistical analysis

The data were presented as mean ± standard deviation, GraphPad Prism software was used to perform the statistical analysis (one-way analysis of variance (ANOVA) and Fisher's LSD post-hoc test ($p < 0.05$)).

Results and discussion

As the elderberry fruits are edible and used in several food products, in this study we choose three cell lines that aim to represent the main tissues of contact after ingestion, a cell model of intestinal tract (Caco-2; human colorectal adenocarcinoma), a hepatocyte cell line, HepG2 (human hepatic carcinoma) cells, aiming to analyse the potential effect after intestinal absorption, and the RAW 264.7 (mouse macrophages) cells aim to assess the systemic effect as macrophages are present in every tissue and are the common cell model to assess the anti-inflammatory activity promoted by plant or fruit extracts.

First, aiming to select the range of non-cytotoxic concentrations of elderberry extracts [total elderberry extracts (E), obtained from each of the 3 cultivars, and its fractions obtained by SPE separation, designated by: rich-phenolic extract (M) and non-phenolic extract (H)], Caco-2, HepG2 and RAW 264.7 cells were exposed for 24 h or 48 h to different concentrations (up to 1 mg/mL) of each extract (Fig. S1). We have observed, for the three cell lines, 100 % cell viability for cells exposed to concentrations up to 500 μg/mL (Fig. S1). However, Caco-2 cells' viability was not affected by any of the extracts at 750 μg/mL (100 % cell viability; Fig. S1-B), for both exposure times. Thus, a range of non-cytotoxic concentrations was set for the next experiments, in which we aim to assess beneficial effects of various elderberry extracts while observing no cytotoxic effect.

Elderberry extracts dose-dependently show anti-inflammatory potential

RAW 264.7 cells were pre-exposed to the various extracts, for 24 h, prior to LPS stimulation (see methods for details). LPS, by binding to its receptor (TLR4) at RAW 264.7 cells' plasma membrane, activates a cascade of intracellular events that results in the production of nitric oxide (NO), a pro-inflammatory mediator, that is released into the incubation media (Silva et al., 2020). The anti-inflammatory activity is thus assessed by the capacity of extracts to reduce the NO release by RAW 264.7 cells. As shown in Fig. 1, all elderberry extracts (up to 100 μg/mL), from the three cultivars, dose-dependently reduced the production and release of NO, in comparison to control cells, although at different extents. This indicates that the different elderberry extracts

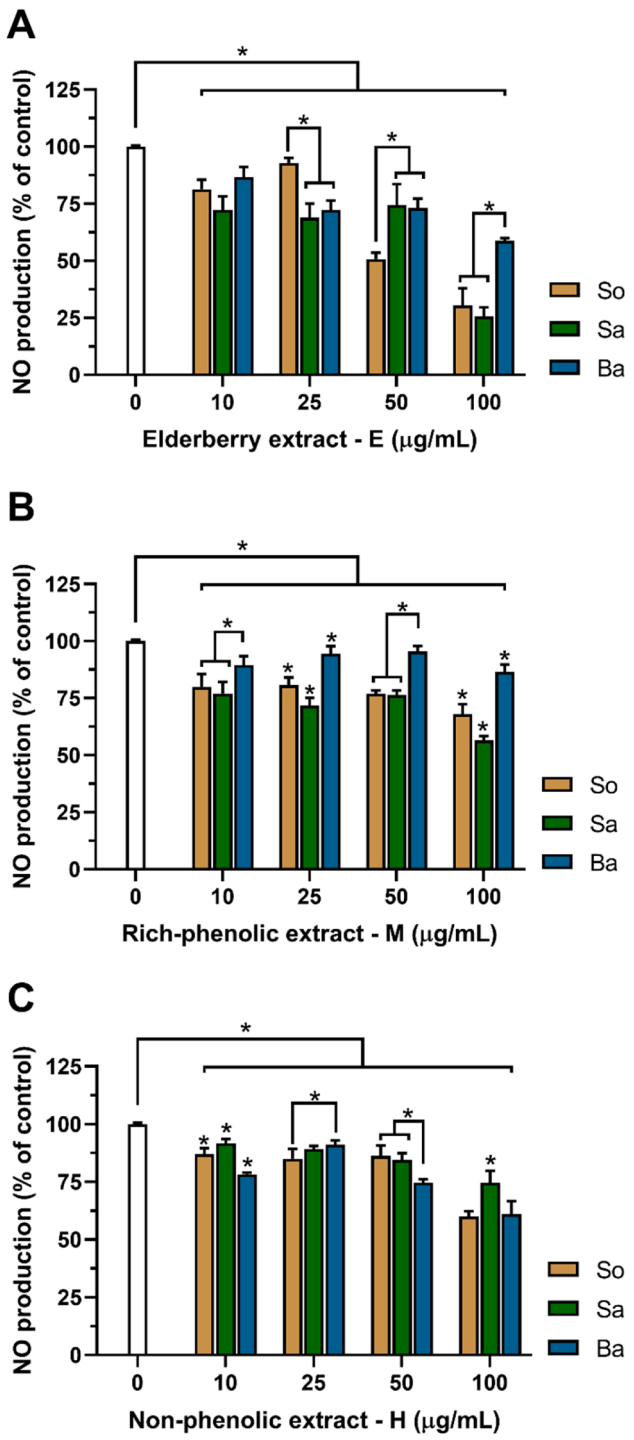

Fig. 1. Anti-inflammatory activity promoted by elderberry extracts in LPS-stimulated RAW 264.7 cells, assessed by NO release quantification. Effect of elderberry crude extract, E (**A**); rich-phenolic extract, M (**B**); and non-phenolic extract, H (**C**); from Sabugueiro (So), Sabugueira (Sa) and Bastardeira (Ba) cultivars. Results are presented as mean ± SD (n = 3 independent experiments, each one in quadruplicates), set as % of control (LPS-exposed cells). Significant statistical differences between the control and samples are denoted by an "*", and between samples at same concentration by an "*" over square brackets, when ($p < 0.05$).

have anti-inflammatory effect.

However, the rich-phenolic extracts (M) of Bastardeira cultivar (Ba), at 25 and 50 μg/mL did not significantly reduce NO release (Fig. 1B), in comparison to control ($p > 0.05$). Rich-phenolic extracts of the three cultivars at 100 μg/mL produced the higher anti-inflammatory effect, being the LPS-induced NO production after pre-exposure to extracts around 67.99 % (Sabugueiro), 56.48 % (Sabugueira) and 86.55 % (Bastardeira), in relation to the control. Thus, rich-phenolic extracts from Sabugueira cultivar at 100 μg/mL was more effective than the other ones at the same concentration ($p = 0.0002$ and $p < 0.0001$, Sabugueiro and Bastardeira, respectively).

Pre-exposure to non-phenolic extract (H; Fig. 1C), reduced LPS-stimulated NO production in comparison to control cells ($p < 0.05$). For Sabugueiro, cells pre-exposed to H extract at 100 μg/mL produced 60.09 % of NO (~40 % reduction), a lower amount than that produced by the other concentrations ($p < 0.0001$). While, cells pre-exposed to Sabugueira H extract, presented 75.65 % of NO production at 100 μg/mL (Fig. 1C; a reduction of ~25 %). On the other hand, Bastardeira H extract at 100 μg/mL lowered the production of NO to 61.08 %, comparing to control ($p < 0.0001$) (reduction of ~40 %). Comparing the cultivars, Sabugueira (100 μg/mL) produced lower anti-inflammatory activity than Sabugueiro and Bastardeira ($p < 0.0001$), while no significant differences were found between Sabugueiro and Bastardeira.

Regarding the elderberry extracts before de SPE separation (E; Fig. 1A), all tested concentrations, from the three cultivars, decreased the LPS-stimulated NO production ($p < 0.05$). Sabugueiro extract, at 50 and 100 μg/mL showed high inhibition effect on NO production, being the NO release, at 100 μg/mL, about 30.44 % of control ($p < 0.0001$). Whereas Sabugueira extract at 100 μg/mL reduced LPS-stimulated NO production in about 75 % (NO production ~25.57 % of control ($p < 0.0001$)). Bastardeira, at 100 μg/mL, significantly reduced LPS-stimulated NO production (NO production of ~58.78 % of control, $p < 0.0001$), but not so high as the other cultivars. As observed in Fig. 1, in general, extracts from Bastardeira cultivar produced the lower anti-inflammatory activity.

Since the SPE separation allows to obtain in the same time, one extract rich in phenolic compounds using methanol for the elution (M) and an extract eluted with water (H) rich in polar compounds, as sugars and organic acids (see Ferreira et al. (2020b) and Table S1 for phytochemical composition), these results suggested that the anti-inflammatory effect of elderberry extracts was not produced alone by the polyphenol compounds or by the polar compounds presented in the aqueous fraction but, instead, was resulted from a synergetic effect between all compounds present in elderberries' matrix, as the elderberry extracts (E), i.e. before SPE separation, showed higher capacity to reduce LPS-stimulated NO production, showing the higher anti-inflammatory activity (Fig. 1A). In general, the M extract produces higher anti-inflammatory activity than the H extract (Fig. 1B and C, respectively).

Ho et al. (2017) reported that elderberry and elderflower extracts of *Sambucus nigra* L. (cultivar "Sampo", grown in Norway), as well as its different polyphenols and their metabolites, produce anti-inflammatory activity by decreasing NO production in LPS-activated RAW 264.7 cells and in dendritic cells (murine dendritic D2SC/I cells). In macrophages, using different elderberry extracts obtained with different solvents (dichloromethane, 96 % ethanol, 50 % ethanol, 0.5 % trifluoroacetic acid in methanol, water at 50 °C, water at 100 °C and press juice extract), they found that water extracts and 50 % ethanol extract did not produce anti-inflammatory activity, but pressed juice and the acidified methanol extract (both at 100 μg/mL) inhibited LPS-induced NO release by 30 % and 50 %, respectively (Ho et al., 2017). Moreover, polyphenol compounds (up to 100 μM) dose-dependently inhibited NO release, having cyanidin, cyanidin-3-glucoside and cyanidin-3-sambubioside identical effects at 100 μM (about 60 % to 70 % inhibition of NO release). Regarding the other flavonoids, quercetin produced stronger inhibitory effect than quercetin-3-glucoside, quercetin-3-rhamnoside and quercetin-3-rutinoside (80 % inhibition vs 50 %), and isorhamnetin and isorhamnetin-3-rutinoside produced 40.5 to 50 % inhibition, at 100 μM. Chlorogenic acid, one of the major phenolic acids present in elderberries, produced about 51.5 % inhibition of LPS-induced NO release

(Ho et al., 2017). These compounds are also present in the elderberry extracts of the three Portuguese cultivars (see Table S1), and the high content in cyanidins and in quercetin, as well as in their derivatives, may explains the anti-inflammatory activity produced by the extracts, and that a synergistic effect may occur as the whole extract was the most potent in inhibiting the LPS-induced NO release (Fig. 1).

Olejnik et al. (2015) reported that, digested elderberry extracts (by simulated gastrointestinal digestion), in a co-cultured of Caco-2 cells and LPS-stimulated RAW 264.7 cells, produced anti-inflammatory effect by down-regulating the pathways that produce interleukin-1β (IL-1β), IL-6, TNF-α, cyclooxygenase-2 (COX-2), PGE_2, and NO.

Karlsen et al. (2007) reported that anthocyanins present in Medox capsules (containing purified anthocyanins isolated from bilberries and blackcurrant, with at least 40 – 50 % of cyanidin-3-glucoside and delphinidin), inhibited LPS-induced activation of transcription factor nuclear factor-kB (NF-kB) in human monocytes cell line (U937-3 cell line), and in a clinical trial (n = 120 subjects) conducted to evaluate the effect of Medox supplementation for 3 weeks (300 mg/d), they found that Medox supplementation decreased the plasma concentration of different inflammatory mediators, such as IL-8, IFNα (inducer of NF-kB activation), IL-4 and IL-13, which supports the anti-inflammatory effect.

Several other studies demonstrated anti-inflammatory activity of cyanidin-3-glucoside, which represents one of the major anthocyanin present in elderberries. Cyanidin-3-glucoside significantly inhibited the expression and secretion of TNFα and IL-6 by a mechanism that involves the suppression of IkBα phosphorylation and degradation, in LPS-stimulated THP-1 cells (Zhang et al., 2010), and in LPS-induced RAW-264.7 cells (Min, Ryu, & Kim, 2010). Cyanidin-3-glucoside also inhibited the expression of iNOS and COX-2, decreasing NO and PGE_2 levels in THP-1 cells stimulated with LPS (Q. Wang et al., 2008). Cyanidin-3-glucoside enhanced the expression and transcriptional activities of nuclear receptor peroxisome proliferator activated receptor γ (PPARγ) and liver X receptor α (LXRα) in LPS-activated THP-1 (Q. Wang et al., 2008).

Li et al. (2014) evaluated the anti-inflammatory potential of crude extracts, as well as, the fractions obtained by SPE separation, named anthocyanin-rich fraction and des-anthocyanin fraction from seven berries (red raspberry, mulberry, black raspberry, triple crown, shawnee, chester and kiown). Their results pointed to a dose-dependent inhibition of NO production, compared with control, in LPS/IFN-γ-induced RAW 264.7 cells, being the crude extracts more effective in lowering NO production than the anthocyanin-rich fraction or the des-anthocyanin fraction (Li et al., 2014), the same trend as we observed in this work (Fig. 1).

Thus, results (Fig. 1) are in accordance with those mentioned above, pointing that elderberries promote anti-inflammatory activity. Part of the anti-inflammatory activity might be due to cyanidin-3-glucoside, one of the main anthocyanin present in elderberry extracts (Table S1), moreover, other compounds presented in elderberries also contribute showing a synergetic effect that allowed the decrease of LPS-induced NO production, as observed in elderberry crude extract (before SPE separation; Fig. 1A). Overall the best extracts are the crude extract (E) and the phenolic-rich extract (M).

Potential protection of elderberries against tert-butyl hydroperoxide (t-BOOH)

In a recent study, we have reported that the three elderberry cultivars have strong *in vitro* antioxidant activity, assessed by ABTS radical scavenging, being extracts from Bastardeira cultivar the ones that, on average, produced higher antioxidant activity (Ferreira et al., 2020b). Based on this, here we aimed to evaluate the potential protection offered by elderberry extracts against a potent pro-oxidant insult, exerted by *t*-BOOH, on Caco-2 and HepG2 cells. Considering the cell viability results (Fig. S1), the fact that the phenolic compounds' profile is identical between cultivars, differing only according to the extract type (Table S1) and that phenolic compounds have a major contribution to antioxidant activity; cells were pre-exposed to concentrations up to 200 μg/mL of elderberry extract (E) and of rich-phenolic extract (M) for 4 h, followed by 16 h incubation with fresh medium before being exposed to *t*-BOOH, for additional 4 h, prior to cell viability assessment (see methods, for details).

The protective effect of elderberry extracts against *t*-BOOH was performed in Caco-2 cells, as these represent a model of intestinal epithelial barrier and ingested foods that protect this barrier will contribute to a healthy gut. Fig. 2A and B, shows that exposure of Caco-2 cells to 250 μM *t*-BOOH resulted in cell damage, reducing Caco-2 cell viability to ~19 % comparing to the untreated control. Caco-2 cells pre-treatment with 50 μg/mL of rich-phenolic extracts (M) or of crude elderberry extracts (E) was effective in protecting cells against oxidative damage promoted by *t*-BOOH, having the three cultivars identical effect (Fig. 2A). E-extracts from the three cultivars, at 50 μg/mL, exert identical protective effect, being Bastardeira effectiveness slightly lower ($p = 0.0431$ and $p = 0.0083$, So and Sa, respectively).

As shown for Bastardeira cultivar (Fig. 2B), dose-dependent effect was observed, with 200 μg/mL of Bastardeira M−extract promoting ~70 % of cell viability, which is a high protecting effect but identical to that observed at 50 μg/mL (Fig. 2A and B). As observed (Fig. 2A and B), all concentrations of rich-phenolic extracts (from the 3 elderberry cultivars) significantly protected Caco-2 cells against *t*-BOOH damage, as cell viability is significantly higher than that of *t*-BOOH control ($p < 0.0001$), even at 12.5 μg/mL of Bastardeira M−extract. Regarding Caco-2 cells pre-treatment with Bastardeira cultivar elderberry extract (E), also dose-dependent effect was found, however the lower concentrations, 12.5 and 25 μg/mL, were not effective in protecting the cells against *t*-BOOH (Fig. 2B, dashed bars) and effects at 50 μg/mL and 200 μg/mL were not statistical different ($p > 0.05$).

The protective effect of elderberry extracts against *t*-BOOH was notable in HepG2 cells (Fig. 2C and D). As observed for Caco-2 cells, exposure of HepG2 cells to 250 μM *t*-BOOH resulted in loss of cell viability, being cell viability about 17 % of untreated control (Fig. 2C and D, red bars). HepG2 cells pre-exposed to 50 μg/mL elderberry extracts (M and E) showed a significant protection against *t*-BOOH, being sufficient to get cell viability above 70 % of control (Fig. 2C), being the rich-phenolic extract (M) the most effective. Concerning the cultivars, rich-phenolic extract (M) from Sabugueiro showed the highest cellular anti-oxidative protection, followed by Sabugueira and by Bastardeira, which allowed a cell viability of ~93 %, 84 % and 70 %, as compared to untreated control, respectively. The effect of different concentrations of Bastardeira M−extract (Fig. 2D), showed identical of concentrations up to 50 μg/mL (~75 % cell viability), but 200 μg/mL increased significantly the cell viability (to ~87 % of untreated control).

Concerning HepG2 pre-exposure to Bastardeira E-extract (Fig. 2D), as seen to Caco-2 cells (Fig. 2B), a dose-dependent protection effect against *t*-BOOH is observed, however 200 μg/mL were necessary to produce the same protection effect as 50 μg/mL of Sabugueiro or Sabugueira E-extracts (Fig. 2C). From this we may infer that elderberries from Sabugueiro and Sabugueira cultivars have a higher potential to protect cells against oxidative stress. Fig. 2E shows that pre-exposure to Bastardeira M or E extracts protects HepG2 cell morphology.

These results demonstrated that cells pre-treatment with elderberry extracts before being subject to *t*-BOOH prevented oxidative injuries. Results highlighted the important role of phenolic compounds against oxidative stress, which was accompanied and resulted in cell viability improvement, observed at the lowest concentration of rich-phenolic extract (Fig. 2B and D).

As far as we know, the only study available on the literature aiming to evaluate the cellular protection against oxidative stress, was performed with Rubini® (Rubini, Iprona AG Via Industria LANA, Italy; a commercially available pure elderberry extract) using bovine aortic endothelial cells and hydrogen peroxide (H_2O_2) as oxidative insult (Youdim, Martin, & Joseph, 2000). Bovine aortic endothelial cells were pre-treated with Rubini® (up to 0.5 mg/mL) for 4 h, followed by 2 h of

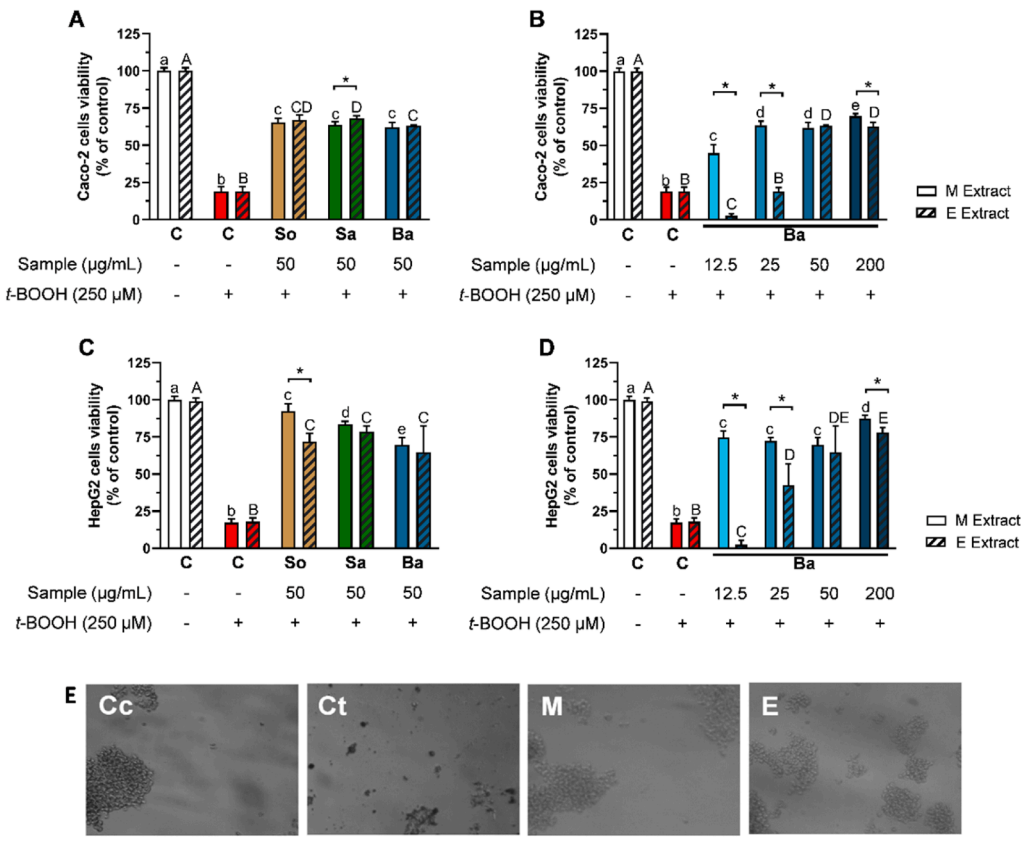

Fig. 2. Elderberry extracts protect Caco-2 (**A and B**) and HepG2 (**C and D**) cells against oxidative insult promoted by 250 μM *t*-BOOH. **A** and **C**: Cells were pre-exposed to 50 μg/mL of elderberry extracts: rich-phenolic extract (M) and crude elderberry extract (E); from Sabugueiro (So), Sabugueira (Sa) and Bastardeira (Ba) before being exposed to 250 μM *t*-BOOH. **B** and **D**: Cells were pre-exposed to different concentrations of Bastardeira (Ba) elderberry M and E extracts before being exposed to 250 μM *t*-BOOH. **E**: Effect of extracts on HepG2 cell density, photos at 100x amplification of: *Cc* – untreated control cells; Ct – control cells exposed to 250 μM *t*-BOOH; M – cells exposed to 50 μg/mL of Ba M extract; E - cells exposed to 50 μg/mL of Ba E extract. In panels **A** to **D**; the "C" denotes control cells. Results are presented as mean ± SD (see methods for details). Means followed by the same letter are not statistically different ($p < 0.05$). Lowercase letters compare the means of M extract; Capital letters compare the means of E extracts; and the "*" compares M with E extract at the same concentration, when $p < 0.05$.

incubation with H_2O_2 (75, 150 and 300 μM), and results showed a dose-dependent cytotoxic effect of H_2O_2 that was dose-dependently prevented by Rubini® extract, although at 300 μM H_2O_2 the cytoprotection was identical to all extract concentrations (Youdim et al., 2000).

Thus, this work reports for the first time the protective effect of elderberry extracts from three cultivars against the oxidative stress promoted by *t*-BOOH in Caco-2 and HepG2 cells. However, some studies using other kind of berries, grapes or isolated phenolic compounds, were made in different cell lines. S. Wang et al. (2016) evaluated the protective effect of extracts from grape by-products (up to 10 μg/mL) and some of its phenolics (gallic acid and syringic acid) on *t*-BOOH-induced oxidative stress (400 μM for 3 h) in Caco-2 cells, observing that pre-treatment for 20 h with grape phenolic extract, gallic acid, and syringic acid significantly reduced the cytotoxic effect induced by *t*-BOOH. Pre-treatment of HepG2 cells with cranberry juice and cranberry phenolic-rich extract was effective in reducing cells' damage induced by *t*-BOOH (400 μM, 2 h), assessed by lactate dehydrogenase (LDH)-leakage assay (Martín et al., 2015), and cranberry phenolic-rich extract was more effective in preventing GSH depletion and in reducing ROS than cranberry juice; contributing to a higher protective potential (Martín et al., 2015). Protective effect against *t*-BOOH-induced oxidative stress in HepG2 cells was shown to the wild edible berry (*Corema album* (L.) D. Don) extracts (acetone, ethyl acetate and water extract) which were essentially composed by hydroxycinnamic acids and, as minor compounds, by several flavonoids and stilbenes. HepG2 cells pre-treated with different extracts for 20 h (up to 40 μg/mL), before the oxidative insult (400 μM *t*-BOOH. for 3 h), showed reduced cell death, ROS content, GSH depletion, and oxidative damage in proteins and lipids (León-González et al., 2012). Identical results were reported for HepG2 cells pre-treated, for 3 h, with a functional juice rich in phenolic compound (26 % of juice grape, 2 % of cherry, 0.6 % of blackberry, 0.6 % of blackcurrant and 1 % of raspberry juice) which increased cell survival and decreased lipid peroxidation after exposure of cells to the pro-oxidants *t*-BOOH and H_2O_2 (24 h) (García-Alonso, Ros, & Jesús Periago, 2006).

Quercetin, a flavonoid present in all extracts (Table S1), was used to pre-treat HepG2 cells and demonstrated to be effective in preventing the oxidative damage promoted by *t*-BOOH, as it abolished LDH leakage, prevented the decrease of GSH and the increase of MDA (malondialdehyde), thus decreased ROS generation (Alía et al., 2006).

As observed, elderberry extract's components protect HepG2 and Caco-2 cells against *t*-BOOH-induced oxidative stress, thus we further explored the cellular mechanisms involved in this protection. As Caco-2 cells grow in monolayer, instead of small aggregates as do HepG2 cells, it is easier and more accurate to assess morphological changes from Caco-2 cells. Thus Caco-2 cells were chosen for the next assays.

Elderberry extracts protect Caco-2 cells against t-BOOH oxidative insult

Extracts preserve cell morphology, prevent DNA damage and mitochondrial depolarization as assessed by fluorescence microscopy

Since elderberry extracts were efficient in protecting cells against *t*-BOOH-induced oxidative damage we further explored their protective effect at other cellular parameters; namely against *t*-BOOH-induced changes in cell morphology, DNA damage and mitochondrial depolarization. Caco-2 cells were pre-exposed to extracts and further to 100 or 250 μM *t*-BOOH (as described above), then were incubated with Hoescht 33342 (to visualize the nucleus) and with JC-1 (indicator of mitochondrial potential) fluorescent probes. Hoechst 33342 probe permeates living cells and binds to DNA, specifically in adenine–thymine-rich regions, it is used to assess the health of cells since stained chromatin, when condensed/fragmented stains more brightly compared to healthy cells that show a dim blue fluorescence (Crowley, Marfell, & Waterhouse, 2016; Martins-Gomes et al., 2019). JC-1 is a lipophilic and cationic dye, that permeates the cell and accumulates into the mitochondria in a potential-dependent way, thus healthy cells with polarized

mitochondria accumulate JC-1 in mitochondria forming JC-1 aggregates that emit red fluorescence. Whereas, in depolarized mitochondria [when mitochondrial membrane potential is low (<100 mV)], JC-1 is released into cytoplasm, being in the monomeric form, emitting green fluorescence (Pernelle et al., 2011; Reers et al., 1995).

Exposure of Caco-2 cells to 100 μM *t*-BOOH did not reduce cell viability (Fig. S2), it did not significantly change cellular morphology (bright field), neither damaged DNA integrity but shifted JC-1 fluorescence, as more cells have green fluorescence as compared with untreated control cells (Fig. S3). On the other hand, Caco-2 cells exposed to 250 μM *t*-BOOH, which significantly decreased cell viability (Fig. 2), show altered morphology, with shrinkage, induced-DNA damage, and loss of mitochondrial membrane potential (Figs. 3, 2nd row).

As Caco-2 cells' viability was similar upon exposure to elderberry extracts (at 50 μg/mL; from the three cultivars), and as similar cell morphology and effects on DNA and mitochondria potential were observed, only results for rich-phenolic extract (M) and elderberry extract (E) from Sabugueira are shown in Fig. 3 (M−Sa and E-Sa, respectively). Pre-treatment of cells with rich-phenolic extract (M−Sa) or with elderberry extract (E-Sa) followed by exposure to 100 μM *t*-BOOH did not alter cell morphology, but seems to reduce the green fluorescence of JC-1 (Fig. S3). However, Caco-2 cells pre-treatment with M or E extract followed by exposure to 250 μM *t*-BOOH (M−Sa + 250 μM or E-Sa + 250 μM, respectively) showed a clear beneficial effect, comparing with cells exposed to 250 μM *t*-BOOH (Fig. 3). Indeed, cell morphology is preserved, the number of bright blue fluorescent cells is reduced and a shift towards increase in red/green fluorescence of JC-1 is observed, comparing to 250 μM *t*-BOOH exposure (Fig. 3). Thus, elderberry extracts protect Caco-2 cells against oxidative damage produced by exposure to 250 μM *t*-BOOH which might be reflected in

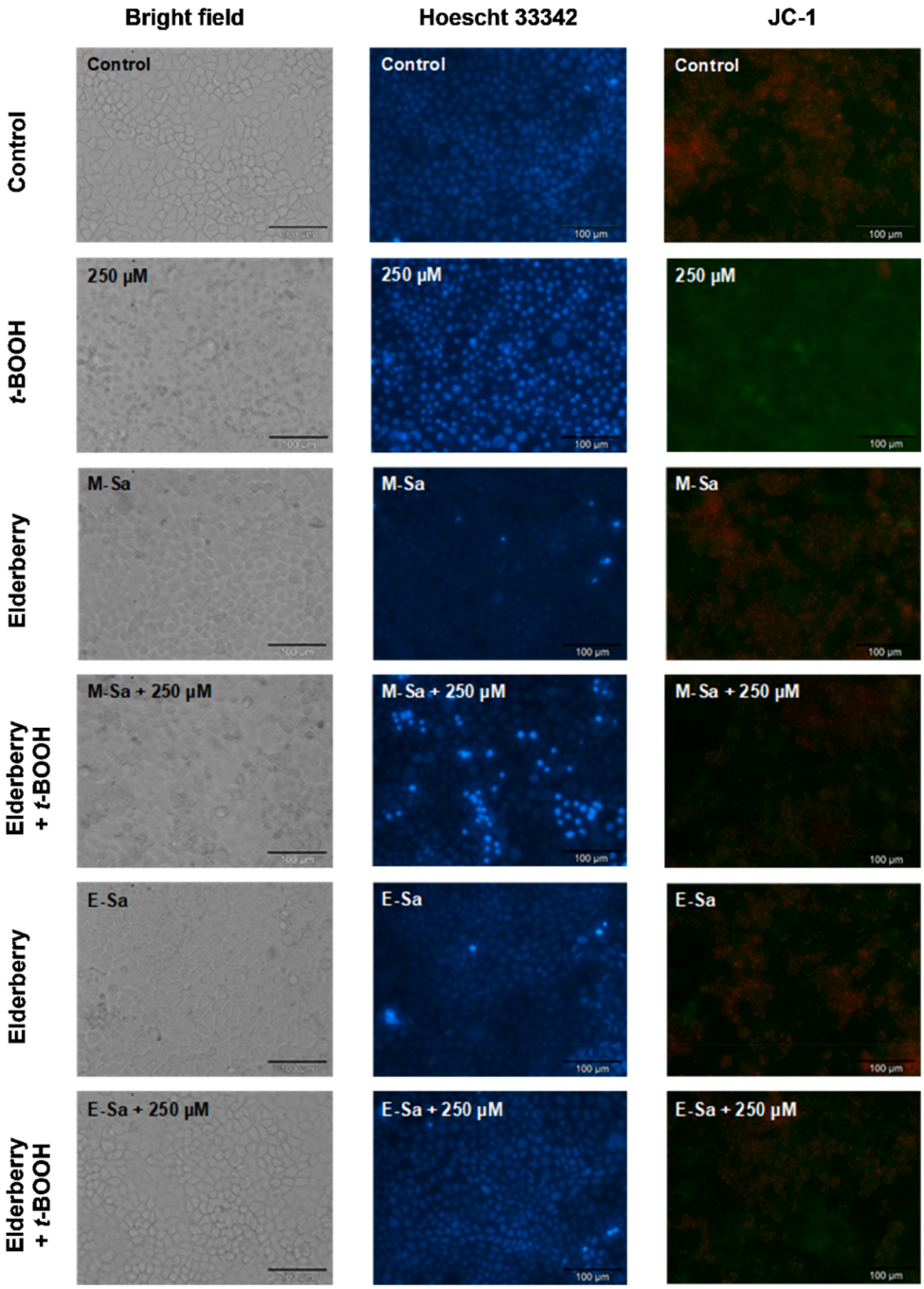

Fig. 3. Effect of rich-phenolic extract (M) and elderberry extract (E) from Sabugueira cultivar in protecting Caco-2 cells against *t*-BOOH-induced damage. Cells were photographed to access morphology (bright field); DNA damage (Hoechst 33342 fluorescence) and mitochondrial depolarization (JC-1 fluorescence), after pre-exposure (or not; control cells) to elderberry extract (50 μg/mL) and then exposed to 250 μM *t*-BOOH, as indicated in figure (see methods for details). Photos were taken using a magnification of 200x, scale bar: 100 μm.

intracellular levels of ROS and GSH, which were further analysed by flow cytometry.

Elderberry extracts protect Caco-2 cells against oxidative stress as assessed by flow cytometry

Oxidative stress markers, such as intracellular levels of ROS, GSH and lipid peroxidation (LP), were assessed by flow cytometry, in Caco-2 cells exposed to 100 or 250 μM *t*-BOOH after pre-exposure to 50 μg/mL of elderberry extracts (see methods).

Fig. 4A shows the protective effect of elderberry E and M extracts, from the 3 cultivars, against the *t*-BOOH-induced increase in intracellular ROS, in Caco-2 cells, using DCFDA as fluorescent probe and flow cytometry analysis. Cells exposed to 100 μM and to 250 μM *t*-BOOH present a MFI (mean fluorescence intensity) of ~2- and ~8-times higher than non-exposed (negative) control cells ($p < 0.05$), respectively, validating the effectiveness of *t*-BOOH as an oxidative agent. First, by its own, elderberry extracts (M and E-extracts) do not induce significant changes in ROS content, comparing to non-exposed control, confirming its safety and correlation with non-cytotoxic effect observed through Alamar Blue assay (Fig. S1-B) and as observed in dot-plots of cells exposed to E-So and M−So (Fig. 4C left panels) the % of viable cells (left-lower quadrant) is maintained. Cells pre-treated with M and E extracts, followed by 100 μM *t*-BOOH, reduced ROS content comparing to positive (*t*-BOOH) control, to emphasize cells pre-treated with E-So extract that present ROS content similar to non-exposed control cells, however differences are not statistical significant (Fig. 4A). To note that, M−Ba

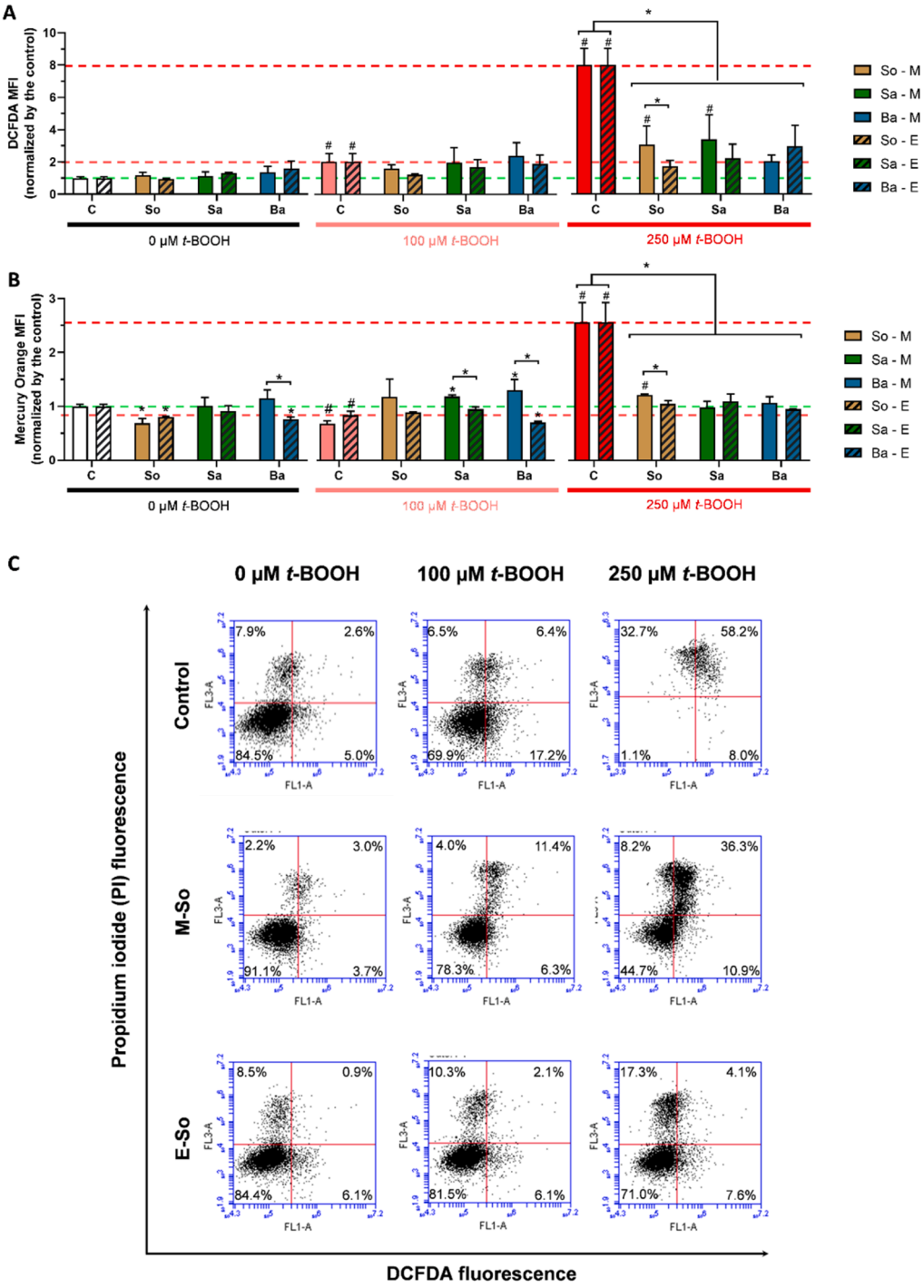

Fig. 4. Elderberry extracts protect Caco-2 cells against *t*-BOOH-induced oxidative stress. Caco-2 cells were subjected to flow cytometry analysis to access oxidative stress markers, after pre-exposure to elderberry extracts (M and E extracts) followed by exposure to 100 or 250 *t*-BOOH μM, as denoted (see methods for details). **A**: DCFDA MFI (mean fluorescence intensity) as a measure of intracellular reactive oxygen species (ROS); **B**: Mercury Orange MFI, as a measure of intracellular glutathione (GSH) content; **C**: Flow cytometry dot-plots showing Caco-2 cells' distribution in DCFDA/PI double staining and antioxidant activity against *t*-BOOH-induced oxidative damage. Dot-plots of control cells and of cells exposed to M−Sa and E-Sa and to *t*-BOOH. Lower left quadrant: $DCFDA^-/PI^-$; lower right quadrant: $DCFDA^+/PI^-$; upper left quadrant: $DCFDA^-/PI^+$; upper right quadrant: $DCFDA^+/PI^+$. Data are expressed as mean ± SD (n = 3 independent experiments). Statistical different means in relation to respective sample in the absence of *t*-BOOH are denoted by # ($p < 0.05$). Means statistical different from negative control are denoted as "*" ($p < 0.05$), and an "*" over a square bracket compares those samples.

and M−Sa extracts were not able to decrease ROS content, comparing to 100 μM *t*-BOOH-exposed cells. On the other hand, all extracts were able to significantly reduce ROS content induced by 250 μM *t*-BOOH (Fig. 4A, rightmost panel), supporting the increased cell viability observed above (Fig. 2A,B). Again, E-So extract is the most effective in preventing the 250 μM *t*-BOOH-induced increase in ROS, as these cells 4-fold less content in ROS than positive control (Fig. 4A), which is followed by M−Ba extract.

Flow cytometry dot-plots obtained for DCFDA/PI double staining (Fig. 4C), shows that negative control presents the cell populations distributed mainly over the lower-left quadrant, representing negative cells to both DCFDA and PI staining. PI is used to evaluate the cells membrane integrity, since PI does not permeate the membrane of healthy cells, being only found intracellularly in cells where the formation of membrane pores has occurred (Narayani, Saravanan, Ravindran, Ramasamy, & Chitra, 2019). Exposure 100 μM *t*-BOOH shifted cells' distribution towards the lower-right quadrant (positive staining for DCFDA, negative to PI), indicating an increase in ROS content but no changes in membrane integrity, and also to the upper-right quadrant indicating cells with significant increase in ROS and loss in cell membrane integrity can be found. When exposed to 250 μM *t*-BOOH, cell distribution shifted to the upper quadrants (90.9 % of the cells), where cells only positive for PI increase ~4.14-times and cells with double staining increase ~22.38-times. Regarding Caco-2 cells pre-treated with M−So and E-So extracts, it can be seen that cells present a control-like cell distribution, confirming the non-cytotoxic effect (Fig. S1), as well as the normal ROS content (Fig. 4A). When challenged with 250 μM *t*-BOOH, cells pre-treated with E-So, present 71 % of healthy cells (double negative staining), compared to 1.1 % of healthy cells in 250 μM *t*-BOOH positive control. Whereas, M−So extract was less effective in protecting cells against *t*-BOOH-induced oxidative stress, but still maintaining 44.7 % of cells with double-negative staining, an increase of ~40.6-times, when compared to 250 μM *t*-BOOH positive control (Fig. 4C). However, cells pre-exposed to M−So extract prior to 250 μM *t*-BOOH, showed higher percentage of cells positively stained for DCFDA or double positive staining, when compared to those pre-exposed to E-So extract.

As expected, GSH content in cells exposed to *t*-BOOH is significantly reduced, comparing to non-exposed control (Fig. 4B). GSH represents cells' major source of reducing capacity, being a major intervenient in the cellular antioxidant system, acting essentially in the elimination of free radicals (Queiroz et al., 2017). Therefore, GSH content decrease in Caco-2 cells exposed to 100 μM *t*-BOOH is a response to the oxidative agent. Cells pre-treated with elderberry extracts (M and E), but not exposed to *t*-BOOH, show slight changes in GSH content, while exposure to M−So, E-So and E-Ba decreased GSH content ($p < 0.05$), the other extracts did not statistically changed GSH content. In response to 100 μM *t*-BOOH, Caco-2 cells pre-treated with E-Ba extracts are the only ones showing a significantly reduced GSH content ($\underline{p} < 0.05$), as depicted in Fig. 4B. Exposure to 250 μM *t*-BOOH, clearly raises GSH levels (Fig. 4B) as a mean of cell survival to counteract the increase in ROS levels (Fig. 4A), extracts with antioxidant activity reduce ROS levels and thus GSH levels are maintained close to negative control. M−So extract highlights as the only extract able to induce a significant increase in GSH content when compared to the non-exposed control (Fig. 4B).

These results suggest that different antioxidant mechanisms might be promoted by the different elderberry extracts, since pre-treatment of cells with the different extracts induced different responses in Caco-2 cells, leading to the observed balance between ROS and GSH, although all extracts present antioxidant potential. Also, as Caco-2 model's the intestinal barriers, ingestion of elderberries (or food products containing elderberries) will expose the intestinal cells to their bioactive compounds and will exert a protective role. However, studies concerning the phytochemicals profile change during digestions and exposure to gut microbiota are needed to assess the amount of phytochemicals reaching the intestinal epithelium.

Regarding lipid peroxidation (LP) assessment (Fig. 5), DPHE-FITC MFI is inversely proportional to the lipid peroxidation occurrence, as when the probe reacts with lipid peroxidation products it loses the fluorescent moiety, and thus loss of fluorescence is observed. Extracts *per se* did not produce lipid peroxidation (Fig. 5, leftmost panel). In all cells pre-treated with elderberry extracts (M and E extracts) and then challenged with 100 *t*-BOOH, LP is at the level of *t*-BOOH-treated cells, and slightly above the negative control (white bars). When cells are challenged with 250 *t*-BOOH, LP is evident (red bars; Fig. 5) which might be the result of the 8-fold increase in ROS (Fig. 4A). It is remarkable to observe that both So extracts strongly prevented LP, followed by both Sa extracts. Concerning Ba extracts, only E-Ba prevented LP induced by 250 *t*-BOOH (Fig. 5).

Taking into account the results obtained, it was expected that cells exposed to 250 μM *t*-BOOH exhibit increased LP, as reported in other studies (Sohn, Han, Lee, & Hwang, 2005). Moreover, phenolic compounds present in grape by-products (Domínguez-Perles et al., 2016; S. Wang et al., 2016), as well as in cranberry extracts (Martín et al., 2015), were effective in preventing GSH depletion, reducing intracellular ROS, and LP against injuries induced by *t*-BOOH, in HaCaT, Caco-2 and HepG2 cells, respectively. In this way, it is clear the beneficial effect of elderberry extracts protecting Caco-2 cells against t*t*-BOOH-induced injuries. Also, anthocyanin rich extracts were effective in preventing oxidative stress induced by H_2O_2, in HaCaT cells, by reducing ROS content and by restoring mitochondrial membrane potential (Molagoda, Lee, Choi, & Kim, 2020), as here observed. These data also highlight a powerful antioxidant role of elderberries, preventing GSH depletion and intracellular ROS production, and protecting cells against oxidative stress, as evidenced by the protection against *t*-BOOH. However, this data resulted from *in vitro* assays, which have the intrinsic limitations, thus further experiments are needed to assess the uptake and bioavailability of these extracts' compounds through biological barriers in order to extrapolate to in vivo.

Conclusions

This work showed for the first time the anti-inflammatory potential

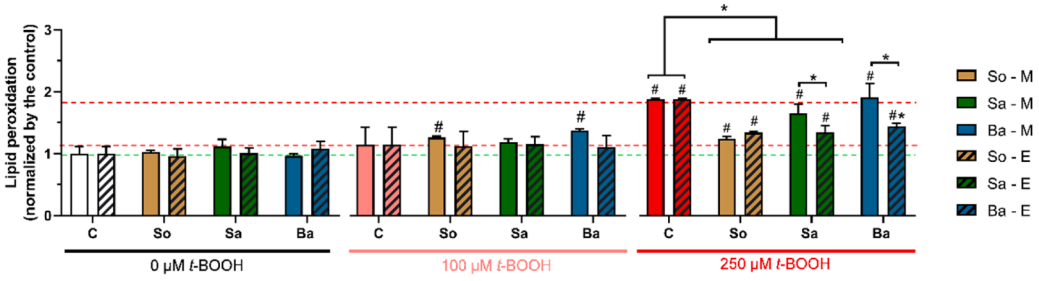

Fig. 5. Elderberry extracts protect Caco-2 cells against *t*-BOOH-induced lipid peroxidation (LP). Caco-2 cells were subjected to flow cytometry analysis to assess lipid peroxidation, using DHPE-FITC probe, after pre-exposure to elderberry extracts (M and E) before being challenged with *t*-BOOH, as denoted (see methods for details). Results were normalized to control (non-exposed cells) and are presented as mean ± SD (n = 3). Statistical different means in relation to respective sample in the absence of *t*-BOOH are denoted by # ($p < 0.05$). Means statistical different from respective control are denoted as "*" ($p < 0.05$), and an "*" over a square bracket compares those samples.

of Portuguese elderberries using LPS-stimulated RAW 264.7 macrophage cells. Among the three cultivars, Sabugueira was the most effective in reducing LPS-induced NO production. Dose-dependent anti-inflammatory activity was observed, for all extracts. Being the crude elderberry extract (before SPE separation), the one showing higher anti-inflammatory potential, highlighting the synergetic effect among the compounds, such as phenolic compounds and polar compounds, as sugars. Elderberry extracts also showed potent action in the antioxidant defence against cytotoxic *t*-BOOH concentrations, very remarkable in HepG2 cells, which prevented the oxidative damage and recovered the cell viability to non-cytotoxic levels. However, the antioxidant protection of elderberries was efficient preventing GSH depletion and in decreasing intracellular ROS induced by high cytotoxic concentrations of *t*-BOOH in Caco-2 cells. These results showed the strong healthy benefits of Portuguese elderberries, mainly of Sabugueiro cultivar, presenting a strong anti-inflammatory and antioxidant potential, which might be due to the phytochemical profile (e.g. anthocyanin, phenolic acid) of elderberry however further analysis are needed to correlate the phytochemicals with specific metabolic pathways. These two bioactivities are crucial to reduce or prevent some disorders associated with inflammatory status and ROS production, and in this way, elderberries might represent a good strategy to prevent the unbalance defence mechanism generated by an excess of NO and ROS production. Thus elderberries consumption will contribute to the cellular oxidative status regulation and will contribute to a better health in general, elderberries could be considered a functional foods or source of functional ingredients however studies concerning the absorption and bioavailability of elderberry phytochemicals are still needed in order to better correlate these results with effective health benefits.

CRediT authorship contribution statement

Sandrine S. Ferreira: Investigation, Data curation, Writing – original draft. **Carlos Martins-Gomes:** Investigation, Data curation, Validation, Writing – review & editing. **Fernando M. Nunes:** Conceptualization, Funding acquisition, Resources, Writing – original draft, Writing – review & editing. **Amélia M. Silva:** Conceptualization, Investigation, Data curation, Validation, Funding acquisition, Resources, Writing – original draft, 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 data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fochx.2022.100437.

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