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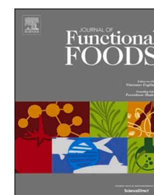

Comparative studies on the anti-neuroinflammatory and antioxidant activities of black and red goji berries

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ABSTRACT

Goji berries have several bioactivities and are consumed as food or used in folk medicine. In this study, the methanolic extracts from red and black goji berries were compared regarding their anti-inflammatory and antioxidant activities. Black goji berries extract has revealed significantly higher anti-inflammatory activity, in either biochemical assays or via decreasing the release of nitric oxide and inhibiting the gene expression of pro-inflammatory cytokines in LPS-stimulated BV2 microglial cells, in comparison with the red goji berries extract. Black goji berries' extract has also revealed a significantly higher antioxidant activity, in comparison with the red goji one. Inflammation and related oxidative stress have been brought to light as underlying mechanisms in several chronic diseases, e.g. neurodegenerative diseases. Our results show that these goji berries, particularly the black ones, can have the potential to be a source of pharmacologically active compounds in diseases with an inflammatory and oxidative background.

1. Introduction

Red and black goji berries are the fruits of two shrub plants of the Solanaceae family, *Lycium barbarum* (*L. barbarum*) and *Lycium ruthenicum* (*L. ruthenicum*), respectively. These berries have been used as traditional medicinal foods in China and other Asian countries for centuries, with the fresh or dried fruits being usually consumed as food, and the fresh leaves being used as infusion (Potterat, 2010; Xu, 2013). Despite the close phylogenetic relationship between these two species, the red and black goji berries exhibit distinct phenotypic profiles, including their shape, size, colour, taste, nutritional value and pharmacological properties (Zhao et al., 2020). Their phenotypes also differ significantly in metabolic terms, namely in the content of fatty acids, phenols and antioxidant capacities, which are much higher in black goji berries (Islam, Yu, Badwal, & Xu, 2017; Zhao et al., 2020). Red goji berries have an ancient history and due to their intensive research, they are nowadays widely and globally used as a "superfood", being considered a functional food supplement (Jiang, Fang, Leonard, & Zhang, 2021; Qian, Zhao, Yang, & Huang, 2017; Wetters, Horn, & Nick, 2018; Yao, Heinrich, & Weckerle, 2018). The consumption of black goji berries is less common, having been studied at a much lesser extent.

Several studies have reported that goji berries, namely the red ones, have several biological activities, including antioxidant (Magiera & Zaręba, 2015; Skenderidis et al., 2019; Song, Gao, & Xu, 2014), hypoglycemic and hypolipidemic (Luo, Cai, Yan, Sun, & Corke, 2004; Zhu et al., 2013), anti-tumour and cytoprotective (He, Yang, Jiao, Tian, &

Abbreviations: LrND, non-digested *Lycium ruthenicum* berries extract; LrD, digested *Lycium ruthenicum* berries extract; *LbND*, non-digested *Lycium barbarum* berries extract; *LbD*, digested *Lycium barbarum* berries extract.

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Zhao, 2012; M. Zhang et al., 2005), anti-inflammatory (Kang, Xue, Du, & Zhu, 2017; Nardi et al., 2016), neuroprotective (Cao, Du, & Hei, 2017; Hu, Qu, Chu, Li, & He, 2018; Yu et al., 2005) and immunomodulation (Chen et al., 2015; Zhang et al., 2011). These biological activities can derive from the presence of high content of several functional components, such as polysaccharides, carotenoids and phenolics compounds including phenolic acids and flavonoids. The later are believed to be pharmacologically important, mainly regarding the antioxidant activity (Islam et al., 2017; Ma et al., 2019; Qian et al., 2017).

The comparison of the phenolic profiles and antioxidant capacities of the red and black goji berries has been addressed by Islam et al. (2017), using several biochemical assays. The phenolics and antioxidant capacities were found to be higher in the black berries extract than in the red ones. To our knowledge, this is the only scientific study addressing the comparison between these Lycium berries regarding their biological activities. Indeed, there are far fewer studies addressing the black goji berries and specifically concerning their anti-inflammatory potential. Among these, Peng *et al.* (2014) demonstrated that a *L. ruthenicum* polysaccharide significantly inhibited the LPS-induced NO production and the mRNA expression of iNOS and several pro-inflammatory cytokines in RAW264.7 cells. In addition, a crude anthocyanins extract from black goji berries has been found to restore several physical signs related to the dextran sodium sulfate-induced colitis in mice and also to decrease the expression of proinflammatory cytokines (Peng et al., 2019). Specifically regarding the neuroinflammation and neuroprotective activities of Lycium berries, the few studies that have been reported so far, are related to *L. barbarum* (Cao et al., 2017; Hu et al., 2018; Yu et al., 2005). Cao et al. (2017) suggested a link between oxidative stress and neurotoxicity, with *L. barbarum* polysaccharide exhibiting protective effects against neurotoxicity by upregulating Nrf2/HO-1 signalling. Indeed, the central nervous system is particularly susceptible to ROS-induced damage and numerous studies have demonstrated that oxidative stress is closely associated with the development of neurodegenerative diseases, reviewed in Uttara *et al.* (2009). Yu et al. (2005) demonstrated that an aqueous extract of red goji berries significantly protected neurons against Aβ peptide toxicity, underlining its neuroprotective potential against neuronal degeneration. In addition, Hu et al. (2018) have also demonstrated the neuroprotective effects of a *L. barbarum* water extract in an Alzheimer's disease mice model. To our knowledge, there are no studies addressing the anti-neuroinflammatory or neuroprotective activities of black goji berries.

In view of the above, and stressing that black goji berries have been poorly investigated, this study aims to assess and compare the antioxidant and anti-inflammatory activities of black and red goji berries, regarding specifically their anti-neuroinflammatory activities. The linked antioxidant activity and the effect of the *in vitro* simulated gastrointestinal digestion on the phenolics contents and in the anti-inflammatory and antioxidant activities of black and red goji berries extracts, have been also assessed.

2. Materials and methods

2.1. Chemicals and reagents

For the biochemical assays, DMSO (dimethylsulfoxide, Hybri-Max™, sterile-filtered, BioReagent ≥ 99.7% purity), Folin–Ciocalteu reagent, sodium carbonate, galic acid (≥97% purity), quercetin, (≥95% purity), DPPH (2,2- diphenyl-1-picrylhydrazyl), trolox (6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid, 97% purity), iron(II) sulfate heptahydrate (99% purity), ferrozine (3-(2-Pyridyl)-5,6- diphenyl-1,2,4-triazine-4′,4′′-disulfonic acid sodium salt, 97% purity), EDTA (Ethylenediaminetetraacetic acid, ≥98.5% purity), TPTZ (2,4,6-tripyridyl-s-triazine, ≥98% purity), iron (III) chloride hexahydrate (97% purity), ascorbic acid (≥99% purity), sodium nitrite, aluminium chloride hexahydrate, PMS (Phenazine methosulfate 99% purity), sulfanilamide, NED (N-1-napthylethylenediamine dihydrochloride, ≥ 98% purity), egg yolk homogenates, BHT (2,6-di-*tert*-butyl-4- hydroxytoluene), TBA (thiobarbituric acid 98% purity), SDS (sodium dodecyl sulphate), 1-butanol, hematin porcine, arachidonic acid (≥85% purity), cyclooxygenase 2 (human), COX-2 inhibitor (Methyl [5-methylsulfonyl-1-(4-chlorobenzyl)-1H-2-indolyl]carboxylate) and TMPD (N,N,N′,N′-Tetramethyl-p-phenylenediamine, 99% purity), were purchased from Sigma (Darmstadt, Germany). Acetate buffer was purchased to Panreac (Barcelone, Spain) and phosphoric acid 85% to Riedel-de Haën (Fisher Scientific, Portugal). For the *in vitro* digestion assay, alpha-amylase from human saliva, pepsin from porcine gastric mucosa, pancreatin from porcine pancreas and bile extract porcine were purchased from Sigma (Darmstadt, Germany). For the cell culture assays, high glucose DMEM (Dulbecco's Modified Eagle's Medium), FBS (Fetal bovine serum), trypsin-EDTA solution, antibiotic–antimycotic solution, t-BHP (*tert*-Butyl hydroperoxide), LPS (lipopolysaccharides from *Escherichia coli*, O111:B4 Sigma), DCFDA (2′,7′-dichlorofluorescein diacetate) and MTT (3-[4,5- dimethyl-thiazol-2-yl]-2,5-diphenyl tetrazolium bromide, ≥ 97.8% purity), were purchased from Sigma (Darmstadt, Germany). GRS Total RNA Kit – Blood & Cultured Cells, Xpert cDNA Synthesis Mastermix and Xpert Fast SYBR Green Master Mix were purchased from GrisP (Porto, Portugal). Primers for mouse interleukin 1β (IL-1β), interleukin 6 (IL-6), inducible nitric oxide synthase (iNOS), prostaglandin-endoperoxide synthase 2 (COX-2) and tumor necrosis factor (TNF) were purchased from StabVida (Oeiras, Portugal).

2.2. Preparation of the Lycium berries extracts

L. ruthenicum and *L. barbarum* were cultivated in China, at Urumqi and Xi'an regions, respectively. The berries were harvested, lyophilized, powdered, and submitted to methanolic (80%) extraction, for 5 days, in darkness, at 25 °C. After filtration, the solvent was removed with a rotary evaporator, at 45 °C, and freeze-dried to obtain a powdered extract. This extract was resuspended in DMSO at 100 mg/ml final concentration and stored at −20 °C. When necessary this solution was diluted to be used in the subsequent assays.

2.3. In vitro digestion of the Lycium berries extracts

The *in vitro* gastrointestinal digestion was simulated according to methodology previously described (Koehnlein et al., 2016). Briefly, 3 g of black or red goji extracts were individually mixed with 9 ml of artificial saliva solution containing 2.38 mg/ml Na_2HPO_4, 0.19 mg/ml KH_2PO_4, 8.0 mg/ml NaCl and alpha-amylase (1.5 mg/ml). The pH was adjusted to 6.75 (at 37 °C). This blend was shaken at 150 rpm during 10 min. Subsequently, the pH was adjusted to 1.2 and 9 ml of artificial gastric fluid (3.2 mg/ml pepsin in 0.03 M NaCl) was added. The mixture was then incubated on a shaker, 150 rpm, at 37 °C for 120 min. Lastly, the pH was adjusted back to 6.0, and 1.5 ml NaCl (120 mM), 1.5 ml of KCl (5 mM) and 9 ml of artificial intestinal fluid (10 mg/ml of pancreatin and 11 mg/ml of bile extract in 9 ml of 0.1 M $NaHCO_3$) were sequentially added. The mixture was incubated at 37 °C for 60 min, at 150 rpm. Thereon the obtained digested extract was freeze-dried and stored at −20 °C, for further use.

Following this procedure, four types of extracts were obtained and used in all the subsequent assays: the non-digested *L. ruthenicum* (LrND) and digested (LrD) extracts from *L. ruthenicum* berries (black goji), and the non-digested (LbND) and digested (LbD) extracts from *L. barbarum* berries (red goji).

2.4. Phenol and flavonoid content determination in the Lycium berries extracts

The total phenolic content was estimated using the Folin-Ciocalteu colorimetric method (Islam et al., 2017). Briefly, 10 μl of the Lycium berries extracts ((LrND, LrD, LbND and LbD, 10 mg/ml) were added to 1.4 ml of deionized water and 100 μl of Folin–Ciocalteu reagent. After

the addition of 300 µl of Na_2CO_3 solution (20% w/v), the mixture was left for 120 min incubation in the dark and then, the optical density was measured at 765 nm. The phenolic content was expressed as gallic acid equivalents (GAE, mg GAE/g extract) using a standard curve of gallic acid.

The flavonoid content was measured using a modified colorimetric method (Islam et al., 2017). Briefly, 10 µl of the berries extracts (10 mg/ml) was mixed with 745 µl deionized water, 100 µl 0.05 g/ml $NaNO_2$ and vortexed. After 6 min, 100 µl $AlCl_3{\cdot}6H2O$ solution (0.1 g/ml) were added and vortexed. Six minutes later, 1 ml of NaOH 1 M was added and vortexed; after 15 min, the optical density was measured at 510 nm. The total flavonoid content was expressed as quercetin equivalents (mg quercetin equivalents/ g extract) using a standard curve of quercetin.

2.5. In vitro antioxidant activity

2.5.1. DPPH radical scavenging assay

The radical scavenging activity was measured using the stable DPPH free radical as described elsewhere with slight modifications (Oliveira, Pinho, Fonte, Sarmento, & Dias, 2018). In a 96-well plate, 140 µl of DPPH 400 µM solution (in 100% ethanol) was mixed with 10 µl of several concentrations of the Lycium berries extracts (LrND, LrD, LbND and LbD, up to 1000 µg/ml final concentration). After 1 h in the dark, the absorbance of the solution was measured at 517 nm. The extract concentration that scavenges 50% of the initial DPPH (EC50) was calculated, using Graph Pad Prism software. Trolox was used as reference standard.

2.5.2. Iron chelating activity (ICA)

The chelation of ferrous ions by Lycium extracts was estimated as described before (Oliveira et al., 2018) with slight modifications. Briefly, 50 µl of 0.12 mM $FeSO_4$ was added to 50 µl of several concentrations of the Lycium berries extracts (LrND, LrD, LbND and LbD, up to 1000 µg/ml final concentration). The reaction was initiated by the addition of 50 µl of 0.6 mM ferrozine solution and the mixture was incubated for 15 min at room temperature, in the dark. The absorbance of the solution was thereafter measured at 562 nm. EDTA was used as reference standard. The extract concentration that chelates 50% of the ferrous ion (EC50) was calculated.

2.5.3. Ferric reducing antioxidant power activity (FRAP)

The antioxidant capacity of the extracts regarding the reduction of Fe3+- TPTZ (colourless complex) to Fe2+- TPTZ (blue coloured complex), by the action of electron donating antioxidants at low pH, was performed according with a methodology previously described (Benzie & Strain, 1996). The FRAP reagent containing 2.5 ml 10 mM TPTZ solution (in 40 mM HCl), 2.5 ml 20 mM $FeCl_3{\cdot}6H_2O$ and 25 ml acetate buffer (0.3 M, pH 3.6) (1:1:10), was freshly prepared and warmed to 37 °C. In 96-well plates, 150 µl FRAP reagent was mixed with 15 µl water and 20 µl of Lycium berries extracts samples (LrND, LrD, LbND and LbD, up to 1000 µg/ml final concentration) and incubated at 37 °C for 30 min in the dark. Then, the absorbance was recorded at 593 nm in a microplate reader. The control and the blank contained all the reaction reagents except for the extract sample and the FRAP reagent, respectively, that were replaced by acetate buffer. The reaction was carried out in triplicates. The FRAP values were expressed as µmol of acid ascorbic equivalents per g of sample extract (µmol AAE/ g) according to a standard calibration curve of ascorbic acid with linear range from 1 to 20 µg /ml ($R2 > 0.99$).

2.5.4. Nitric oxide (NO) scavenging activity

The NO scavenging activity was performed as described elsewhere (Silva, Malva, & Dias, 2008) with slight modifications. Briefly, the reaction mixture (200 µl) containing SNP (10 mM, in PBS, pH 7.4) and the Lycium berries extract samples (LrND, LrD, LbND and LbD, up to 1000 µg/ml final concentration), were incubated at 25 °C for 2 h. A control was included in which the extract was replaced by PBS, pH 7.4. Then, 50 µl of Griess reagent (1% (w/v) sulfanilamide, 0.1% (w/v) NED in 2% phosphoric acid) were added and incubated for 10 min, in the dark. Two blanks have been included: the sample blank and the control blank with similar composition of their counterparts but with 2% phosphoric acid instead of the Griess reagent. The absorbance of the chromophore (purple azo dye) was measured at 543 nm. Ascorbic acid has been used as reference standard. EC50 values were obtained as described before.

2.6. Cell culture

BV2 (mouse microglia) cell line was kindly donated by Dr. Annika Höhn (German Institute of Human Nutrition, Germany) and HepG2 (human hepatocytes) was obtained from the American Type Culture Collection (ATCC). They were maintained in culture in 25 cm^2 polystyrene flasks (Falcon) with complete DMEM (supplemented with 10% FBS, 2 mM L-glutamine, 1% antibiotic–antimycotic solution and 1.5 g/L sodium bicarbonate) at 37 °C in a humidified incubator containing 5% CO_2. Cells were sub-cultured once a week using 0.25% trypsin-EDTA and seeded at a density of 1.5×10^6 cells per ml. In all the subsequent assays, DMEM without FBS was used.

2.7. Cell viability/cytoxicity assay

In order to evaluate the potential cytotoxicity of Lycium berries extracts, both BV2 and HepG2 cells were seeded in 96-well culture plates at a density of $1.5x10^6$ cells/ml (200 µl/well) in complete DMEM. Twenty-four hours after plating, the medium was discarded. The cells were then incubated with the berries extracts (LrND, LrD, LbND and LbD) at different concentrations, ranging from 100 to 1000 µg/ml, for 5 h and 24 h. Cells just in DMEM (without the extracts) or with 10 % DMSO in DMEM were used as normal growth and death controls, respectively. Cell metabolic activity (viability) was determined using the MTT assay as described before (Oliveira et al., 2018). The cell viability was expressed as percentage of the control (cells just with DMEM).

2.8. Cytoprotection against t-BHP oxidative insult

The t-BHP is a stable hydroperoxide that in physiological conditions induces an oxidative insult being commonly used as a model for the evaluation of oxidative stress in cells and tissues (Oliveira et al., 2018). In order to evaluate the cytoprotective potential of Lycium berries extracts (LrND, LrD, LbND and LbD) against t-BHP insult, HepG2 cells, seeded in 96-well culture plates, were first pre-incubated with extracts for 2 h (short pre-incubation period) or 21 h (long pre-incubation period), at 100, 250 and 500 µg/ml concentrations, and then co-incubated with t-BHP (0.5 mM) for additional 3 h. Cellular viability was assessed by the MTT assay. HepG2 cells in DMEM (not incubated with the extracts; normal growth), 10 %DMSO in DMEM (death control), and in DMEM with t-BHP (0.5 mM), were used as controls for the cytoprotective assays.

2.9. Intracellular reactive oxygen species (ROS)

Intracellular ROS levels were measured using a DCFDA fluorescent probe. HepG2 cells were seeded in black 96-well plates. After 24 h, the medium was removed and 25 µM DCFDA was added to each well for 30 min at 37 °C. After the removal of the DCFDA, cells were washed with PBS, and pre-incubated with the Lycium berries extracts (LrND, LrD, LbND and LbD), for 2 h. Then, they were co-incubated with t-BHP (0.5 mM) for 3 h. The fluorescence intensity was measured in a fluorescence spectrophotometer (Fluoroskan Ascent™ FL Microplate Fluorometer and Luminometer, ThermoScientific) at excitation and emission wavelengths of 488 and 535 nm, respectively.

2.10. Evaluation of the anti-inflammatory activity of the Lycium berries extracts

2.10.1. In vitro determination of anti-inflammatory activity (COX-2)

COX-2 peroxidase endpoint assay was performed as previously described with some modifications (Mogana, Ten-Jin, & Wiart, 2013). Briefly, 20 μl of the Lycium berries extracts (LrND, LrD, LbND and LbD) were plated in 96-multiwell plates at a final concentration of 500 μg/ml. Then 20 μl of 0.01 U/μL of COX-2 enzyme solution was added, followed by 160 μl of endpoint assay mix solution consisting of 100 μM bovine hematin chloride, 10 mM of arachidonic acid, 17 mM of TMPD, and 1 M of buffer Tris-HCl pH 8.1. The absorbance was measured at 595 nm, after 10 min of incubation at room temperature, in the dark. Inhibition of COX-2 was expressed as percentage (% ICOX-2) relatively to the full enzyme activity (without berries extracts) calculated as follow: % ICOX-2 = $(E - S)/E \times 100$, where E is the full enzyme activity and S the enzyme activity with tested sample extract. Methyl [5-methylsulfonyl-1-(4-chlorobenzyl)-1H-2-indolyl]carboxylate, a selective COX-2 inhibitor, was used as positive control. All measurements were performed in triplicate.

2.10.2. Determination of NO production

The amount of NO released by BV2 cells into the culture medium and converted into nitrite was measured using the Griess reaction. Cells were plated in 12-well plates for 24 h in DMEM, at a density 1.5×10^5 cells/ml (1.5 ml/ well). After treatment with the Lycium berries extracts (LrND, LrD, LbND and LbD), at 100 and 250 μg/ml, for 3 h, NO production was stimulated by adding LPS (1 μg/ml final concentration), and co-incubation for further 20 h. The culture supernatant was then collected and 50 μl were transferred to 96-well plates and incubated with 50 μl of 1% (w/v) sulfanilamide in 2% phosphoric acid for 10 min. After further 10 min incubation with 50 μl of 0.1%, w/v NED in 2% phosphoric acid, the absorbance at 543 nm was measured. Both incubations were carried at room temperature in the dark.

2.10.3. Determination of the expression of pro-inflammatory genes by BV2 cells

2.10.3.1. RNA isolation from BV2 cells and cDNA synthesis. Similarly to the previous assay, BV2 cells were plated in 12-well plates, pre-incubated with the Lycium extracts (LrND, LrD, LbND and LbD, 250 μg/ml) for 3 h and activated by LPS addition (1 μg/ml final concentration). After 20 h, the cells were recovered for RNA extraction using the GRS Total RNA Kit – Blood & Cultured Cells (GrisP) according to manufacturer's instructions, being the samples treated with DNAse to remove any contaminant DNA. The RNA integrity and purity was checked through the ratios 260/280 and 260/230 in a nanodrop (NanoDrop Bioanalyzer ND1000 Delaware, USA) and in a 1% agarose gel. Subsequently, reverse transcription and first-strand cDNA synthesis was performed on a Mastercycler Nexus (Eppendorf, Germany) using 1 μg of each sample RNA with the Xpert cDNA Synthesis Mastermix (GrisP) following the manufacturer's instructions.

2.10.3.2. Reverse transcription-polymerase chain reaction (qRT-PCR). Quantitative real-time PCRs were prepared with Xpert Fast SYBR Green Master Mix (GrisP) and performed in 96-well plates using a CFX96 Touch™ Real-Time PCR Detection System (BioRad). For each sample (biological replicate), qPCR reactions were undertaken in triplicate using 5 μl Master Mix, 300 nmol/L of each primer, 1 μl of diluted cDNA (1:10) and nuclease-free water to a final volume of 10 μl. The following cycler conditions were used: 15 min at 95 °C and 45 cycles of 15 s at 95 °C, 30 s at 55 °C and 30 s at 72 °C. Experiments were analysed with the software Bio-Rad CFX Manager (BioRad) using GAPDH as internal control. After each run, melting curves were performed to check for unspecific and primer dimer amplification. The genes under evaluation were IL-1β, IL-6, iNOS, COX-2 and TNF. For this purpose, specific primers were designed with Quantprime Software (Table S1).

2.11. Statistical analysis

All biochemical experiments were performed independently three times, each with three replica. In cell culture experiments, four independent experiments were carried out, each with four replica. Data was analysed in GraphPad Prism v. 8.0 Software (San Diego, CA, USA). Data are shown as mean ± SD. Results were analysed by one-way ANOVA followed by Tukey post-tests for multiple comparisons. Student's paired *t*-test was also employed when appropriate. Differences were considered significant at $p \leq 0.05$. EC50 values were also calculated in GraphPad via non-linear regression analysis. The correlation between the values of the phenols and flavonoids contents of the extracts and their antioxidant activity (DPPH, ICA, FRAP, TBARS and NO scavenging) were analysed using the Pearson test.

3. Results

3.1. Phenolic and flavonoid content is higher in L. ruthenicum berries extracts

The phenolic (mg GAE/g extract) and flavonoid (mg QE/g extract) contents of the Lycium berries extracts, before (ND) and after *in vitro* digestion (D), are presented in Table 1. The results shown that LrND extract has significant higher phenolic and flavonoid contents than LbND (Table 1). A significant decrease in the phenolic and flavonoid contents was also observed in both LrD and LbD extracts, i.e. following the *in vitro* digestion process (Table 1).

3.2. Antioxidant activities (in vitro biochemical assays) are higher in L. ruthenicum berries extracts and globally they were reduced by in vitro simulated digestion process

A first screening for the evaluation of antioxidant activity of Lycium berries extracts (LrND, LrD, LbND and LbD) was performed by several *in vitro* methods, measuring several relevant activities for the overall antioxidant performance of the extracts. Both the original extracts (non-digested) and after *in vitro* digestion were evaluated and the results are compiled at Table 2. In general, the Lycium extracts have moderate antioxidant activities, being *L. ruthenicum* extracts more effective.

3.2.1. DPPH free radical scavenging activity

The DPPH assay is a widely used technique that evaluate the anti-radicalar activity and is based on the quenching of the stable coloured radical (DPPH). All Lycium berries extracts were studied regarding their scavenging activity on DPPH radicals, being expressed as EC50 values (Table 2). Lower EC50 values were obtained for *L. ruthenicum* berries

Table 1
Total Phenolic and flavonoid content of the LrND, LrD, LbND and LbD methanolic extracts (expressed in mg GAE/g extract and mg QE/g extract, respectively). LrND – non-digested *Lycium ruthenicum* berries extract; LrD – digested *Lycium ruthenicum* berries extract; *LbND* - non-digested *Lycium barbarum* berries extract; *LbD* - digested *Lycium barbarum* berries extract.

Lycium extracts	Phenols (mg GAE/ g of extract)	Flavonoids (mg QE/ g of extract
LrND	64.9 ± 0.4	93.9 ± 1.0
LrD	34.4 ± 0.3	54.7 ± 2.3
LbND	30.0 ± 0.4	24.8 ± 5.4
LbD	18.5 ± 0.4	17.9 ± 2.6

GAE – gallic acid equivalents; QE: quercetin equivalents. Values represent mean ± SD of three representative experiments. Significant differences were obtained between the pairs ND/D ($p < 0.0001$), except for the comparison between LbND and LbD flavonoids ($p = 0.121$).

Table 2
In vitro antioxidant capacities of the Lycium berries extracts.

Assay	LrND	LrD	LbND	LbD
DPPH[1]	246 ± 8[a]	293 ± 24[a]	871 ± 55[b]	967 ± 37[b]
ICA[1]	105 ± 5	135 ± 7	330 ± 16[a]	340 ± 22[a]
FRAP (AAE[2]**)**	176.4 ± 1.1	130.9 ± 1.3	57.6 ± 1.8[a]	55.0 ± 5.0[a]
NO[1]	998 ± 70	1657 ± 23[a]	1673 ± 34[a]	1891 ± 95[a]

LrND – non-digested *Lycium ruthenicum* berries extract; LrD – digested *Lycium ruthenicum* berries extract; *LbND* - non-digested *Lycium barbarum* berries extract; *LbD* - digested *Lycium barbarum* berries extract.
Significant differences were obtained between all the values in the same line ($p < 0.05$), except those signalized with the same letter.
[3]MDAE (malondialdehyde equivalents, nmol/ml).
[1] EC50 values (μg/ml).
[2] AAE (acid ascorbic equivalents, μmol/ g extract at 750 μg/ml extracts concentration).

(black) extracts compared to *L. barbarum* (red) ones. Significant differences were obtained between all the values ($p < 0.0001$) except for the comparison between LrND and LrD ($p = 0.419$) and LbND and LbD ($p = 0.053$), indicating that the digestion of extracts have negligible impact on their ability for scavenging DPPH. Trolox included as a positive control has an EC50 of 2 μg/ml.

3.2.2. ICA assay

Iron chelators are important antioxidants by reducing the amount of available iron, therefore decreasing the quantity of hydroxyl radicals generated by Fenton reaction (Silva et al., 2008). ICA of the Lycium berries extracts (LrND, LrD, LbND and LbD) was assessed using EDTA as reference standard (EC50 = 2 ± 0.1 μg/ml). Significant differences were obtained between all the values ($p < 0.0001$) except for the comparison between LrND and LrD ($p = 0.121$) and LbND and LbD ($p = 0.826$). Following the same pattern, LrND presents the strongest activity among the four extracts and the effect of digestion was marginal (Table 2).

3.2.3. FRAP radical scavenging activity

FRAP assay depends on the ability of antioxidants to reduce Fe(III) to Fe(II) in the presence of TPTZ, which lead to the formation of a deep blue Fe(II)-TPTZ complex (Zhao et al., 2018). This method measures the reducing power of an extract. A standard curve with a range of ascorbic acid of known concentrations was created allowing the determination of FRAP radical scavenging activity of the extracts (as mg ascorbic acid equivalents/ g extract). For the same amount of extract (750 μg/ml), higher FRAP values were obtained for LrND and LrD (176.4 ± 1.1 and 130.9 ± 1.3 respectively) in comparison with LbND and LbD (57.6 ± 1.8 and 55.0 ± 5.0 respectively) (Table 2). Significant differences were obtained between all the values ($p < 0.0001$) except for the comparison between LbND and LbD ($p = 0.5093$).

3.2.4. NO scavenging activity

NO radicals are not very reactive but play important and diverse physiological reactions, including oxidative stress, inflammation, and cell death, related with several human pathologies (Silva et al., 2008). Lycium berries extracts (LrND, LrD, LbND and LbD) were studied regarding to their scavenging activity on NO radicals (Table 2). LrND showed the highest NO scavenging activity (EC50 = 998 ± 70 μg/ml), which was strongly and significantly ($p = 0.0002$) reduced after digestion (LrD, EC50 = 1657 ± 23 μg/ml). LbND has a NO scavenging activity similar to LrD (LbND, EC50 = 1657 ± 23 μg/ml), that was slightly, affected by digestion (LbD, EC50 = 1871 ± 95 μg/ml). Ascorbic acid was used as a positive control for NO scavenging activity with EC50 = 70 ± 2 μg/ml.

3.3. Citoxicity of Lycium berries extracts

Since Lycium berries are widely consumed (mainly the red *L. barbarum* ones), it is important to check their potential cellular toxicity. On the one hand, the liver is the primary organ for xenobiotic detoxification, on the other, brain cells are particularly sensitive to toxicity. Therefore, we used HepG2 (human hepatocytes) and BV2 (murine microglia) cells to check any potential toxicity.

HepG2 and BV2 cells were incubated with the Lycium berries extracts, both non-digested and digested, for 5 h or 24 h, at several concentrations (up to 1 mg/ml), mimicking acute and chronic cell exposure, respectively. In general, the incubation of HepG2 cells with Lycium extracts (both non-digested or digested) did not induced any toxicity up to 500 μg/ml, independently of the time set (Fig. 1A,B). At 1 mg/ml, LrND exert a slight but significant toxicity (13% death) after 5 h of cell exposure (Fig. 1A). This effect increased after 24 h of exposure (at 1 mg/ml extract concentration), particularly with LrND (34% death, Fig. 1B), although LrD, LbND and LbD also showed a slight but significant toxicity (16% death for LrD and LbND and 15% death for LbD Fig. 1B).

BV2 cells were more sensitive to Lycium berries extracts (Fig. 1 C,D). After 5 h of exposure, *L. ruthenicum* berries extracts (both LrND and LrD) induced BV2 cell toxicity at the highest concentration used (1 mg/ml) (22.5% and 9.3% cellular death for LrND and LrD respecively, Fig. 1C). This toxicity increased after 24 h of exposition time, with all extracts showing significant toxicity at 500 μg/ml, specially the non-digested ones (Fig. 1D). LrND remains the most toxic with 60% and 34% of cellular death at 1 mg/ml and 500 μg/ml, respectively. Again, in general, *L. ruthenicum* berries extracts were more toxic then their *L. barbarum* equivalents. The digestion had a tendency for reducing this toxicity, but not statistically significant.

3.4. Lycium berries extracts have cytoprotective potential against t-BHP oxidative insult

Since Lycium extracts proved to have *in vitro* antioxidant activities, we investigated the cytoprotective potential of these extracts against t-BHP induced oxidative stress in HepG2 cells. Lycium berries extracts (LrND, LrD, LbND and LbD) were evaluated for their putative HepG2 cytoprotection against oxidative insult (t-BHP 0.5 mM, 3 h), in two types of pre-incubation regimes: addition of the extract 2 h or 21 h, before the oxidative insult (Fig. 2). Following 2 h pre-incubation with the extracts, a dose dependent cytoprotective effect against t-BHP insult was found, resulting in a 1.3- and 0.8-fold significant viability increase, respectively for LrND and LrD (Fig. 2A, 500 μg /ml). No significant cytoprotection was achieved after 2 h pre-incubation with Lb extracts (Fig. 2A).

After 21 h pre-incubation with the Lycium berries extracts, a much higher and significant cytoprotective effect was achieved for all the four extracts, at the highest concentration (500 μg/ml, Fig. 2B). *L. ruthenicum* berries extracts were more effective in this protection, in comparison to the *L. barbarum* equivalents, resulting in cell viabilities similar to control (without the oxidative insult) for LrND at 250 and 500 μg/ml, and LrD at 500 μg/ml (Fig. 2B). In general, no significant differences were obtained between the non-digested and the digested extracts at the same concentration, except for the pair LbND/LbD at 500 μg/ml.

3.5. Lycium berries extracts decreased ROS levels in HepG2 cells

In order to link the cytoprotective effect of the Lycium extracts against the t-BHP insult (pre-incubation of cells with extracts for 2 h, and then incubation with t-BHP for 3 h) and a putative antioxidant effect, we have evaluated the intracellular ROS levels. Therefore, HepG2 were stained with DCFDA, a fluorogenic dye that measures ROS within the cell. As expected, HepG2 cells under t-BHP insult produced significant higher intracellular ROS amounts (Fig. 3). Pre-incubation of HepG2 cells with the Lycium berries extracts resulted in significant reduction of ROS levels induced by t-BHP insult, except for LbD 250 μg/ml (Fig. 3). Remarkably, LrND and LrD reduced ROS levels to similar values presented by control cells (without insult) with non-significant differences between them. Lb extracts were less efficient and they were not able to

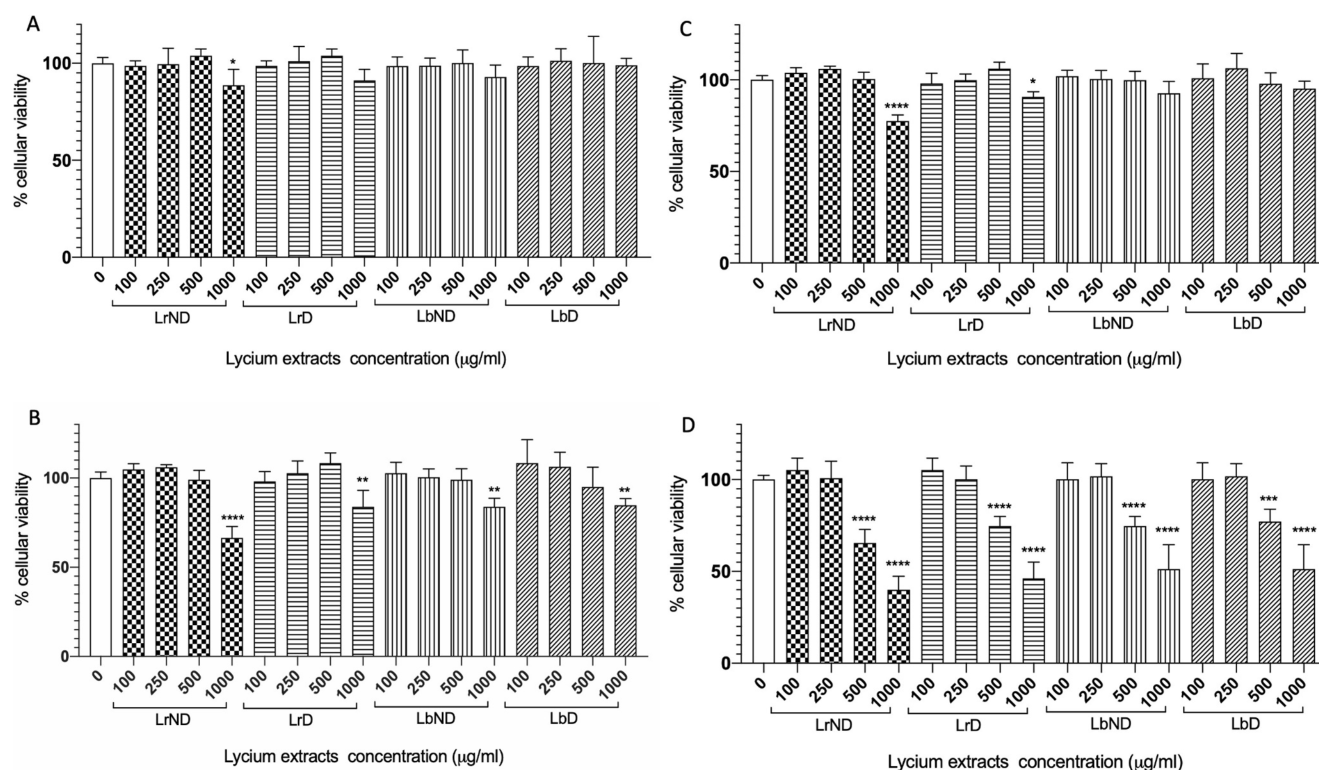

Fig. 1. Cytotoxicity of Lycium extracts on HepG2 cells (A and B) and on BV2 cells (C and D). Lycium berries extracts (LrND, LrD, LbND and LbD, at 100, 250, 500 and 1000 μg/ml), were incubated with HepG2 (A, B) and BV2 cells (C, D) for 5 h (A, C) or 20 h (B, D). Cellular viability was assessed with MTT assay. Each bar represents the mean ± SD of the results obtained in three independent experiments, each with four replica. LrND – non-digested *Lycium ruthenicum* berries extract; LrD – digested *Lycium ruthenicum* berries extract; LbND - non-digested *Lycium barbarum* berries extract; LbD - digested *Lycium barbarum* berries extract. Asterisks mean significantly differences from the negative control (null concentration), as indicated: * P ≤ 0.05; ** P ≤ 0.01; *** P ≤ 0.001; **** P ≤ 0.0001.

reduce ROS to control levels. Nonetheless, LbND at 500 μg/ml ($p < 0.001$) did not show any significant differences from control levels, either. In contrast, LbD at 250 μg/ml showed ROS levels similar to the ones exhibited by insult-cells (Fig. 3). The statistical comparison between the non-digested and the digested extracts, at the same concentration, showed significant differences between LbND and LbD at 250 μg/ml ($p < 0.01$) and 500 μg/ml ($p < 0.05$), indicating a negative impact of digestion in the antioxidant activity of the extracts.

3.6. Anti-inflammatory potential of Lycium berries extracts

3.6.1. Lycium berries extracts show anti-inflammatory activity in the COX-2 peroxidase endpoint assay

There is a reported interdependence between oxidative stress (e.g. ROS production) and inflammation (e.g. cytokine production) (Valacchi, Virgili, Cervellati, & Pecorelli, 2018). A first screening for the evaluation of anti-inflammatory activity of Lycium berries extracts was performed by the COX-2 *in vitro* peroxidase endpoint assay. This assay measures the inhibitory activity against COX-2 enzyme, which is part of the mechanism of action of most nonsteroidal anti-inflammatory drugs. COX-2 is a form of the enzyme that is inducible by cytokines and growth factors, linked to inflammatory cell types and tissues. Lycium berries extracts (LrND, LrD, LbND and LbD) were studied regarding to their COX-2 inhibition activity. The highest COX-2 inhibition was obtained for LrND (circa 80% inhibition), followed by LrD, LbND and LbD (Fig. 4). All conditions were significantly different from each other, except for LrD vs LbND ($p = 0.22$). The COX-2 inhibitor was included as a positive control and had an inhibition of 54% (for 50 μg/ml), under the methodology used.

3.6.2. Lycium berries extracts inhibit NO production by LPS-induced BV2 cells

The anti-inflammatory ability of Lycium berries extracts was assessed in BV2 cells. Briefly, after 2 h-pre-incubation with the extracts, LPS was added to BV2 cells to induce inflammation. NO production was assessed 24 h later by Griess reaction. LPS treatment caused a significant increase in NO release (2.9 fold) by cells compared to basal levels (Fig. 5). All Lycium berries extracts inhibited significantly LPS-induced NO production, in comparison with BV2 cells exclusively incubated with LPS (Fig. 5), with the highest inhibition levels obtained for LrND. Remarkably, no significant differences were obtained between the NO basal levels (control, no LPS insult) and LPS-induced cells treated with LrND at 250 μg/ml (Fig. 5). In general, *L. ruthenicum* berries extracts were more effective then equivalent *L. barbarum* ones, and digestion originated a significant reduction in the anti-inflammatory effect of the extracts. Significant differences between the non-digested vs digested extracts were observed for the pairs LrND/LrD (at 100 μg/ml, $p < 0.05$) and for LbND/LbD (at 100 and 250 μg/ml, $p < 0.0001$).

3.6.3. Lycium berries extracts decrease LPS-induced pro-inflammatory mRNA levels in BV2 cells

To further evaluate the anti-inflammatory effect of the Lycium berries extracts, the expression levels of the pro-inflammatory cytokines genes IL-1β, IL-6 and TNF, and the enzymes iNOS and COX-2 were evaluated upon pre-incubation of BV2 cells with the Lycium extracts and co-incubation with LPS to induce inflammation. As shown in Fig. 6, LPS significantly and strongly increased the expression of IL-β, IL-6, TNF, iNOS and COX-2 genes, in comparison with control cells (cells non-LPS induced). However, when BV2 cells were pre-incubated with the *L. ruthenicum* berries extracts and further co-incubated with LPS, the expression of all these genes significantly decreased (Fig. 6), with the exception of TNF (with LrD). In general, *L. ruthenicum* extracts were

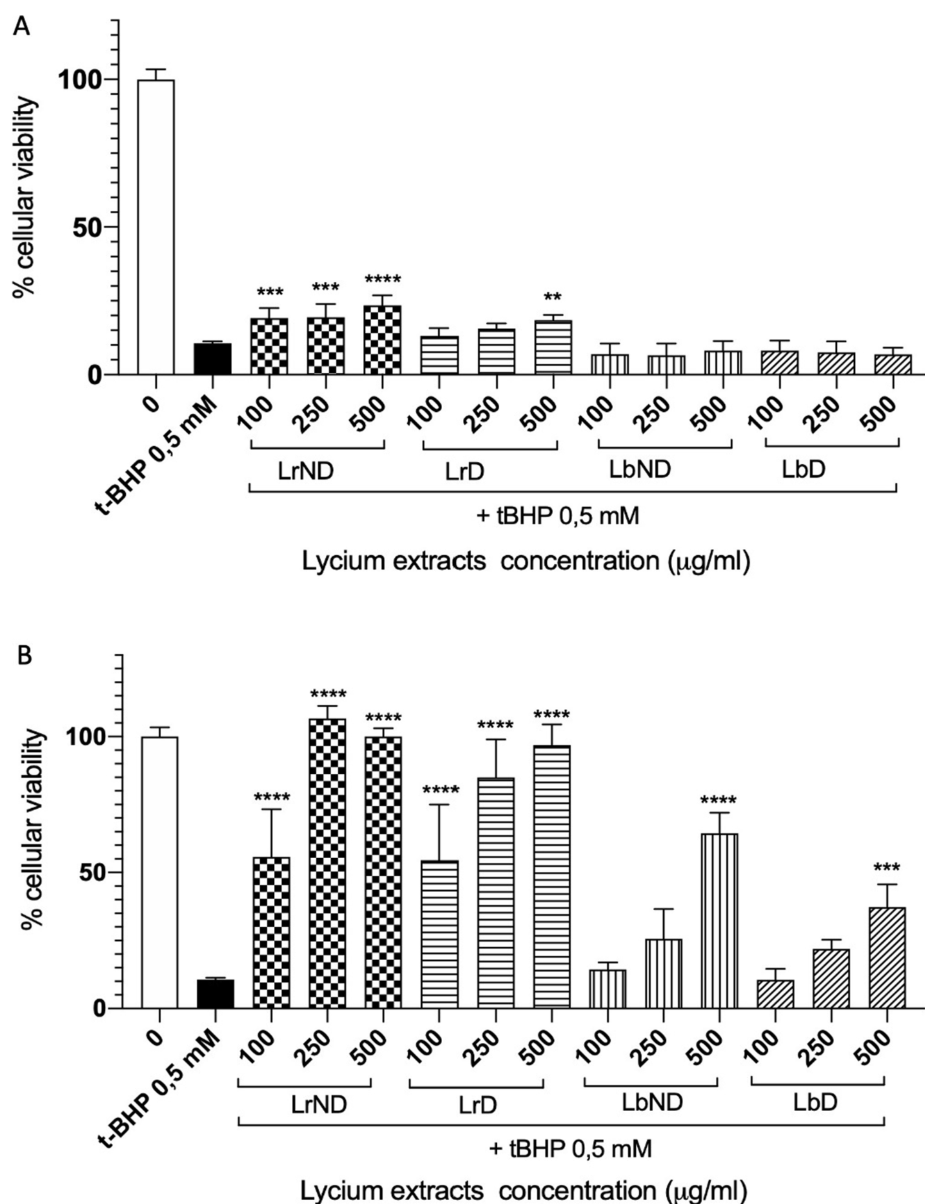

Fig. 2. Cytoptotective potential against t-BHP 0.5 mM. HepG2 cells were pre-incubated for 2 h (A) or for 21 h (B) with the Lycium berries extracts (LrND, LrD, LbND and LbD, at 100, 250 and 500 μg/ml) and co-incubated with t-BHP 0.5 mM for 3 h. Cellular viability was assessed by the MTT assay. LrND – non-digested *Lycium ruthenicum* berries extract; LrD – digested *Lycium ruthenicum* berries extract; LbND - non-digested *Lycium barbarum* berries extract; LbD - digested *Lycium barbarum* berries extract. Each bar represents the mean ± SD of the results obtained in three independent experiments, each with four replica. Asterisks mean significantly differences from the positive control (t-BHP 0.5 mM, cells only incubated with t-BHP 0.5 mM for 3 h), as indicated: ** P ≤ 0.01; *** P ≤ 0.001; **** P ≤ 0.0001.

more effective in the reduction of the expression of the studied genes, compared with *L. barbarum* counterparts. The highest inhibitory levels were obtained for LrND extracts for all the genes tested. Also, digested extracts were in general significantly less effective in the reduction of the genes expression, comparing to their non-digested ones, with LbD being just able to significantly reduce iNOS expression (Fig. 6).

4. Discussion

Inflammation has been brought to light as an underlying mechanism in several chronic diseases, including the neurodegeneratives' (Furman et al., 2019). In fact, chronic neuroinflammation has been considered one of the biological contributors to cognitive aging and plant based extracts have shown to offer interesting pharmacological properties in its prevention (Kure, Timmer, & Stough, 2017). Thereby, the consumption of supplements with one or more plant based extracts or nutraceuticals has been highlighted in the prevention of cognitive aging (Kure et al., 2017). These supplements can act on the several mechanisms that have been shown to be related to cognitive changes across age, including the blood–brain barrier integrity, oxidative stress, microglial activation and chronic low-grade neuroinflammation. Specifically, the over-stimulated microglial cells have been demonstrated to induce detrimental neuronal damage as well as neurodegenerative processes through excess production of multiple pro-inflammatory mediators, such as IL-6, TNF-α, and NO. In addition, iNOS, COX-2 and other enzymes associated with the inflammatory processes may also be induced (Kure et al., 2017).

A few studies have reported that Lycium berries can have neuroprotective effects (Cao et al., 2017; Yu et al., 2005), with all of them addressing to *L. barbarum* berries (red goji) extracts. To our knowledge, there are no studies with *L. ruthenicum* berries (black goji) addressing neuroprotection. In this study, we compare the anti-neuroinflammatory activities of *L. ruthenicum* and *L. barbarum* berries extracts. For that, we use the BV2 microglial cell line, as a suitable model system used in brain inflammation studies (Henn et al., 2009). We demonstrate that *L. ruthenicum* berries extracts' have higher anti-neuroinflammatory activities than *L. barbarum* ones, displayed by the higher outcome in decreasing IL-1β, IL-6, TNF and iNOS and COX-2 gene expression (Fig. 6), and in decreasing NO production in BV2 cells (Fig. 6).

Following LPS insult, IL-6 is the mostly expressed gene in our BV2

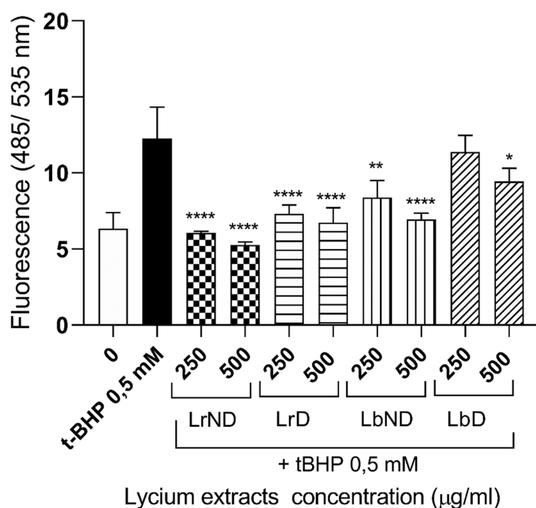

Fig. 3. DCFDA microplate assay. HepG2 cells were labeled with DCFDA (25 μM), pre-incubated with the Lycium berries extracts (LrND, LrD, LbND and LbD, at 250 and 500 μg/ml, for 2 h) and then co-incubated with t-BHP 0.5 mM (3 h). Cells were then analyzed on a fluorescent plate reader with excitation and emission wavelengths of 485/535 nm. LrND – non-digested *Lycium ruthenicum* berries extract; LrD – digested *Lycium ruthenicum* berries extract; LbND - non-digested *Lycium barbarum* berries extract; LbD - digested *Lycium barbarum* berries extract. Each bar represents the mean ± SD of the results obtained in three independent experiments, each with four replica. Asterisks mean significantly differences from positive control (t-BHP 0.5 mM): ****$P < 0.0001$; **$P < 0.01$; * $P \leq 0.05$.

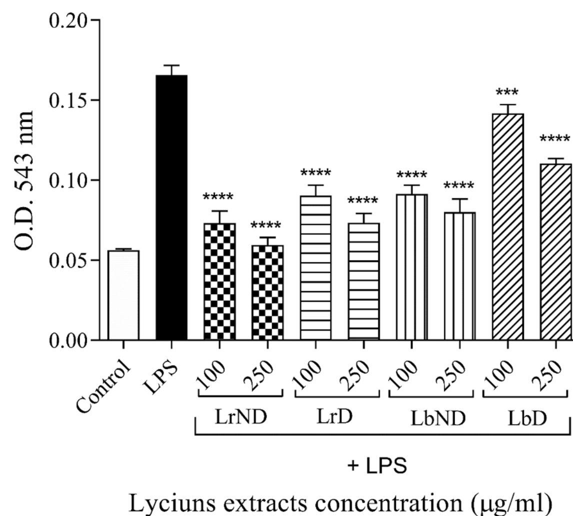

Fig. 5. Anti-inflammatory ability of Lycium extracts. BV2 cells in 12-well plates were pre-incubated (2 h) with Lycium berries extracts (LrND, LrD, LbND and LbD, at 100 and 250 μg/ml), and co-incubated with LPS 1 μg/ml for 18 h. Supernatants were collected, and the NO production in cell supernatants was detected by Griess reaction (O.D. 543 nm). LrND – non-digested *Lycium ruthenicum* berries extract; LrD – digested *Lycium ruthenicum* berries extract; LbND - non-digested *Lycium barbarum* berries extract; LbD - digested *Lycium barbarum* berries extract. Each bar represents the mean ± SD of the results obtained in three independent experiments, each with four replica. Asterisks mean significantly differences from the positive control (LPS, cells incubated exclusively with LPS 1 μg/ml); *** $P < 0.001$; **** $P \leq 0.0001$.

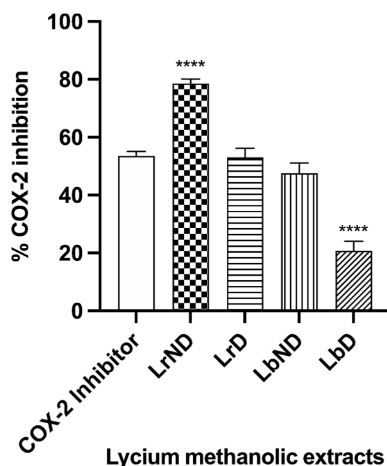

Fig. 4. Inhibition of COX-2 by the peroxidase endpoint assay. Lycium berries extracts (LrND, LrD, LbND and LbD, at 500 μg/ml) or COX-2 inhibitor (50 μg/ml), were mixed with COX-2 enzyme solution and the endpoint assay mix solution containing the substrate arachidonic acid that yield an indirect measure of COX activity. The results are presented as % COX-2 inhibition in relation to the full enzyme activity. LrND – non-digested *Lycium ruthenicum* berries extract; LrD – digested *Lycium ruthenicum* berries extract; LbND - non-digested *Lycium barbarum* berries extract; LbD - digested *Lycium barbarum* berries extract. Each bar represents the mean ± SD of the results obtained in three independent experiments, each with three replica. All conditions were significantly different from each other ($p < 0.001$), using Tukey's multiple comparison test, except for COX-2 inhibitor, LrD, and LbND ($p > 0.05$).

cell assay (Fig. 6). This pleiotropic cytokine that has been assigned with a role in the pathogenesis of inflammatory disorders and in the physiological homeostasis of neural tissue (Rothaug, Becker-Pauly, & Rose-John, 2016). Notably, Alzheimer's disease (AD) is associated with increased IL-6 expression in brain (Rothaug et al., 2016). Additionally, the link between COX-2 upregulation, neuroinflammation and the pathogenesis of neurodegenerative disorders has been also addressed (Guan, Yu, Zou, & Wang, 2019; P; Wang et al., 2014). The signalling pathways by which COX-2 mediates aggravation of AD have been delineated, namely through the reciprocal regulation of IL-1β and amyloid β-protein (Aβ) between glial and neuron cells (Guan et al., 2019; P; Wang et al., 2014). Taken together, these findings demonstrate that COX-2 and IL-1β are activated during the course of AD development and progression and potentially contributes to its pathogenesis (Guan et al., 2019). Since COX-2 is an enzyme form that is inducible by cytokines, being linked to inflammatory cell types, there has been an ongoing effort to identify compounds that might inhibit it (Attiq, Jalil, Husain, & Ahmad, 2018). In the COX-2 peroxidase assay, we found an higher inhibition activity with *L. ruthenicum* berries extracts in comparison to *L. barbarum* ones (Fig. 5), in accordance with the higher ability of *L. ruthenicum* extracts to decrease COX-2 gene expression (Fig. 6, COX-2). Lycium berries extracts, namely from *L. ruthenicum*, had also the ability to decrease IL-1β and others genes expression (IL-6, TNF and iNOS) relevant for inflammation (Fig. 6), being candidates to hamper the described neurodegenerative signalling pathways mediated and sustained by COX-2, IL-1β, and inflammation in general.

The biological activity of phenolic compounds has been shown to be generally suppressed by metabolism (Olthof, Hollman, Buijsman, Van Amelsvoort, & Katan, 2003). In this way, the influence of the gastrointestinal digestion on the food phytochemicals contents and on their bioactivities (namely antioxidant capacity) is being more and more studied. Lycium berries, namely red gogi (*L. barbarum*), are commonly used in the diet. Recent studies have evaluated the effect of the *in vitro* gastrointestinal digestion on the composition and bioactivities of extracts from *L. ruthenicum* (Wang et al., 2019) and *L. barbarum* (Rocchetti et al., 2018) berries'. These studies reported that the *in vitro* simulated gastrointestinal digestion increased the antioxidant activity of *L. ruthenicum* anthocyanins extract (Wang et al., 2019), but decreased the total phenolic content and the antioxidant capacity (ORAC radical scavenging) of *L. barbarum* phenolic extract (Rocchetti et al., 2018). Therefore, in order to anticipate the biological activities of the Lycium

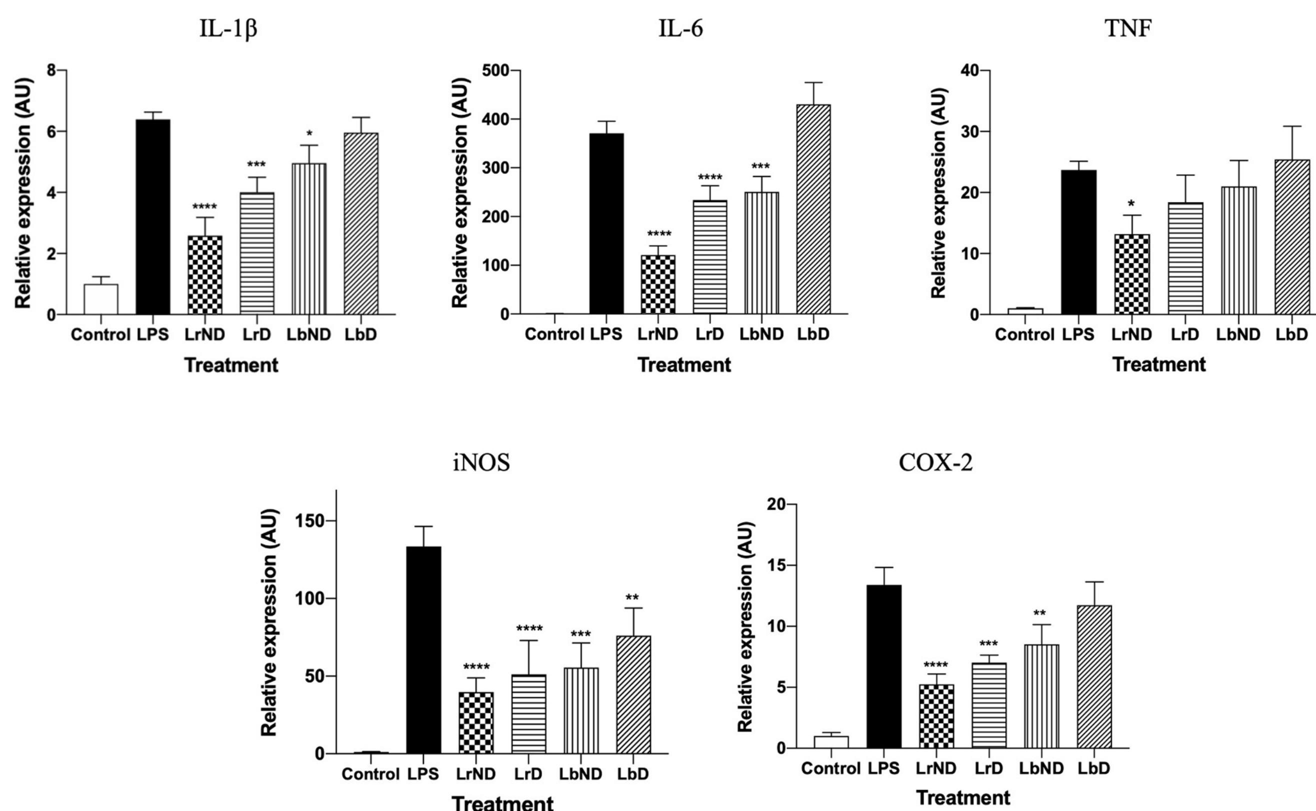

Fig. 6. Inflammatory gene expression in BV2 cells. BV2 cells in 12-well plates were pre-incubated (2 h) with Lycium berries extracts (LrND, LrD, LbND and LbD, at 250 μg/ml) and co-incubated with LPS 1 μg/ml for 18 h. The cells were collected for RNA extraction and RT-PCR. Gene expression (IL-1β, IL-6, TNF, iNOS and COX-2) was calculated using GAPDH expression as an internal control. Non incubated cells (Control) and cells incubated with LPS 1 μg/ml (LPS) for 18 h were used as a negative and positive control, respectively. LrND – non-digested *Lycium ruthenicum* berries extract; LrD – digested *Lycium ruthenicum* berries extract; LbND - non-digested *Lycium barbarum* berries extract; LbD - digested *Lycium barbarum* berries extract. Experiments were analyzed with the software Bio-Rad CFX Manager. Statistical differences are presented vs. positive control (LPS): *$p < 0.05$; ** $p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$.

extracts after human digestion, we performed a simulated *in vitro* digestion and assessed the digested Lycium extracts along with the non-digested ones. We found out that, in general, the digested extracts have decreased anti-inflammatory activity, regarding the expression of pro-inflammatory cytokine and enzymes genes, in NO production and COX-2 inhibition, in comparison with their non-digested counterparts (Figs. 4-6). In addition, the digested extracts also have decreased anti-oxidant activity, in comparison to their non-digested counterparts (Table 2). Nevertheless, we observed that the digested berries extracts still remained fairly effective as anti-inflammatory and antioxidant agents namely the ones from *L. ruthenicum* (Figs. 2-6).

One of the features of the inflammatory response is the generation of a pro-oxidative environment due to the production of high fluxes of pro-oxidant species being "oxinflammation" a novel concept describing this cross talk between inflammatory and oxidative stress mediators (Valacchi et al., 2018). Indeed, oxidative stress has been reported to have a crucial role in the development of different types of human pathologies, including neurotoxicity and neurodegenerative progression through oxidative damage and interaction with mitochondria (Liu, Zhou, Ziegler, Dimitrion, & Zuo, 2017; Silva, Oliveira, Dias, & Malva, 2007). Indeed, neuron cells are particularly vulnerable to oxidative damage due to their high polyunsaturated fatty acid content in membranes, high oxygen consumption, and weak antioxidant defence (Liu et al., 2017). Several studies have previously reported the antioxidant activities of several Lycium species (Magiera & Zaręba, 2015; Skenderidis et al., 2019; Song et al., 2014). These activities are highly relevant for the antioxidant and neuroprotective effects of plant extracts and compounds (Oliveira et al., 2018; Silva et al., 2008). In this study, we showed that *L. ruthenicum* berries extracts had higher cytoprotective activity against t-BHP oxidative stress induced toxicity than *L. barbarum*'s (Fig. 2). This cytoprotective effect can be due to a decreased in ROS levels when HepG2 cells were exposed to t-BHP insult. Our results also showed that both *L. ruthenicum* and *L. barbarum* extracts have antioxidant activities, including scavenging of free radicals and chelation of iron (Table 2). Moreover, the intracellular ROS in HepG2 cells incubated with the Lycium extracts and t-BHP were significantly reduced, up to basal levels (Fig. 3), confirming the antioxidant potential of the extracts.

Lycium berries extracts are rich in phenolics (Islam et al., 2017; Jiang et al., 2021; Mocan et al., 2018). Our Lycium berries extracts also showed high levels of total phenol (TP) and total flavonoids (TF) contents (Table 1), namely the *L. ruthenicum* extracts, in accordance with Islam et al. (2017) results. Most likely, these compounds are partially related with the activities found in our Lycium extracts, as reported for other plants (Oliveira et al., 2018) or even for Lycium species (Islam et al., 2017; Jiang et al., 2021; Mocan et al., 2018). In this study, the digested extracts presented lower antioxidant and anti-inflammatory activities than their counterparts, likely due to the decreased content in phenols and flavonoids (Table 1). Accordingly, phenol and flavonoid contents are higher in LrND, followed by LrD, LbND and LbD (Table 1), suggesting a positive correlation between phenolic compounds and antioxidant and anti-inflammatory activities with *L. ruthenicum* berries extracts holding higher biological activities and higher phenolics contents. Indeed, a positive correlation has been shown between the phenolic content and DPPH, ABTS and FRAP, as reviewed in (Jiang et al., 2021).

In conclusion, we have demonstrated that black goji berries extract had higher phenolic contents and higher anti-inflammatory,

neuroprotective and antioxidant activities than the red goji one and that globally, the *in vitro* gastrointestinal digestion decrease either the phenolics contents or the referred activities. Our results suggest that digestion might modify some chemical entities of the Lycium berries extracts in different molecules, leading to a reduction in biological activities. Nevertheless, the digested extracts still maintain effective levels of antioxidant and anti-inflammatory activities, which might be relevant and validate their human consumption and use. This comparative study brings several scientific insights into the anti-inflammatory and antioxidant activities of both goji berries that can be useful to consumers and stakeholders. *L. barbarum* berries have been indicated as a potential functional food that may be administered to patients (Cao et al., 2017; Hu et al., 2018; Yu et al., 2005). This study highlighted for a better performance of *L. ruthenicum* berries, with no significant cytotoxicity, suggesting a superior potential in preventing or mitigating the oxidative stress and inflammation associated with neurodegenerative disorders. In food industry, Lycium berry products have been divided in two categories: dried whole berry as the final products and berry-integrated products, such as flours, beverages and meat being (Jiang et al., 2021). Thereby, the integration of Lycium berries in several daily products would allow the use of their potential health benefits.

Further studies need to be conducted to find out the compounds responsible for these activities, the underlying mechanism by which Lycium extracts act and the relevant differences in chemical compounds before and after the *in vitro* gastrointestinal digestion.

5. Ethics statement

Not applicable. No studies in humans or animals have been carried out.

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CRediT authorship contribution statement

Vanessa Magalhães: Conceptualization, Writing – review & editing. **Ana Rita Silva:** Writing – review & editing. **Bruna Silva:** . **Xiaoying Zhang:** Writing – review & editing, Supervision, Funding acquisition. **Alberto C.P. Dias:** Conceptualization, Writing – review & editing, Supervision, Funding acquisition.

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.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jff.2022.105038.

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