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Ultrasound-assisted extraction of bioactive compounds from goji berries: Optimization, bioactivity, and intestinal permeability assessment

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ABSTRACT

Lycium barbarum L. berries have a remarkable chemical composition and extensive biological activities, being a valuable component of health and nutraceutical practices. Nevertheless, a deep insight on the intestinal permeation of the pro-healthy bioactive compounds is urgently needed to predict the real effects on human body. This study attempted, for the first time, to optimize the Ultrasound-Assisted Extraction (UAE) of goji berries using a Response Surface Methodology approach and establish the intestinal permeation of the principal pro-healthy compounds. The optimal extraction conditions were a solid:liquid ratio of 8.75 % for 56.21 min, using an intensity of 59.05 W/m^2. The optimal extract displayed a remarkable antioxidant capacity, with LC/DAD-ESI-MS analysis unveiled a diverse phytochemical profile, encompassing different compounds (e.g. glu-lycibarbarspermidine F, 2-glu-kukoamine, rutin, 3,5-dicaffeoylquinic acid). The intestinal co-culture model demonstrated that glu-lycibarbarspermidine F (isomer 2) (73.70 %), 3,5-dicaffeoylquinic acid (52.66 %), and isorhamnetin-3-*O*-rutinoside (49.31 %) traversed the intestinal cell layer, exerting beneficial health-promoting effects.

1. Introduction

Lycium barbarum L. berries, commonly known as goji berries, is a herbal medicinal plant in Asian countries for thousands of years, mostly due to it extraordinary phytochemical profile with outstanding amounts of phenolic compounds (12697.90 mg/100 g fw), organic acids (4461.02 mg/100 g fw), and vitamins, particularly vitamin C (2.39—48.94 mg/100 g fw) (Donno, Beccaro, Mellano, Cerutti, & Bounous, 2015; Zhao & Shi, 2022). This rich bioactive composition is responsible for the well stated goji berries therapeutic properties, namely antimicrobial, antioxidant, anti-inflammatory, and even anti-cancer activities (Teixeira, Silva, Delerue-Matos, & Rodrigues, 2023; Vasantha Rupasinghe, Nair, & Robinson, 2014). Phenolics, particularly phenolic acids, flavonoids, carotenoids, and tannins, are the principal bioactive compounds responsible for these excellent biological activities (Bora, Ragaee, & Abdel-Aal, 2019; T. Islam, Yu, Badwal, & Xu, 2017; D. Pinto, Cadiz-Gurrea, Vallverdu-Queralt, Delerue-Matos, & Rodrigues, 2021; Silva, Costa, Delerue-Matos, Latocha, & Rodrigues, 2021; Zhao et al., 2022). This is the case of feruloylquinic acid and 3,5-dicaffeoylquinic acid, derivatives of caffeoylquinic acid, which demonstrated strong antioxidative properties (S. Islam, Yoshimoto, & Yamakawa, 2006), while glucosidic caffeoylspermidine derivatives have displayed different levels of anti-Alzheimer's disease activity (Zhou et al., 2016).

Skenderidis et al. (2018) attested that the aqueous extract of *L. barbarum* berries prepared by Ultrasound-Assisted Extraction (UAE) increased the levels of glutathione (GSH) and decreased the levels of the lipidic peroxidation marker thiobarbituric acid reactive substance (TBARS), when exposure to muscle cells (P. Skenderidis et al., 2018). In another study, Zhu et al. (2020) reported that the phenolic amides from a non-polysaccharide fraction of an ethanolic *L. barbarum* fruit extract have capacity to modulate the *in vitro* proliferation of B and T-cells and enhance immune cell factors (IFN-γ, IL-2, and IL-10), attesting it

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immunomodulatory effects (Zhu et al., 2020). More recently, the proliferation of human natural killer cells (NK-92) was increased by 61.0 % in the presence of ethanolic goji berries extract (Kwasnik, Lemieszek, & Rzeski, 2021). These exceptional results attracted the attention of food, pharmaceutic, and cosmetic industries (Mocan et al., 2018; Teixeira et al., 2023; Zhao et al., 2022), leading to an increased production of goji berries over the world, with particular focus on China (Donno et al., 2015), Europe (Romania, Italy, Portugal, Bulgaria, and Greece) and Northern America (Mocan et al., 2018).

Despite the wealth findings encompassing goji berries composition and potential health benefits, few is known regarding the intestinal permeation of the different bioactive compounds, a key aspect that directly influences bioavailability and, most important, the *in vivo* biological accomplishments (Sarmento, Andrade, Silva, Rodrigues, das Neves, & Ferreira, 2012). To this end, *in vitro* intestinal co-culture models to assess permeability have surfaced as an economical, straightforward, time-saving, and ethically favorable alternatives, circumventing the ethical concerns of *in vivo* models (Sarmento et al., 2012; Silva, Almeida, et al., 2022).

Selecting an efficient extraction process and the appropriate accoupling conditions, such as temperature, pressure, solvent, and time, is the key strategy to ensure the recovery of bioactive compounds from a natural matrix (Kumar, Srivastav, & Sharanagat, 2021). UAE is a non-conventional and "green" extraction technique extremely popular due to its sustainability and efficacy (Silva, Pinto, Moreira, Costa, Delerue-Matos, & Rodrigues, 2022). This technique operates on the principle that the application of high-intensity sound waves accelerates molecular movement and induces physical disruption of plant tissues through development of forces during acoustic cavitation (Kumar et al., 2021; Silva, Pinto, et al., 2022; P. Skenderidis et al., 2018). Moreover, UAE requires less time and energy-consuming than conventional methods, demanding lower amounts of sample and solvents and employing an equipment easily to handle and applicable to large scale production, being the ideal option for industrial scale-up (Kumar et al., 2021; Silva, Pinto, et al., 2022; Prodromos Skenderidis, Petrotos, Giavasis, Hadjichristodoulou, & Tsakalof, 2017). Consequently, mathematical and statistical software's, such as Response Surface Methodology (RSM), are extremely important for the optimization of UAE processes, allowing to explore the correlations between the independent variables and the responses studied (Kumar et al., 2021; Silva, Pinto, et al., 2022; Prodromos Skenderidis et al., 2017). Nonetheless, to the best of our knowledge, just one study made in 2016 optimized the extraction of goji berries by UAE and used an ultrasound water bath with well-known disadvantageous (Prodromos Skenderidis et al., 2017). Therefore, the aim of this study is to explore how *in vitro* intestinal permeability impacts the bioaccessibility and bioactivity of phytochemical compounds extracted from goji berries using UAE, while assessing its potential as a novel nutraceutical ingredient. RSM was employed to optimize the extraction conditions (solid:liquid ratio, time, and amplitude) and the optimal extract was characterized regarding radical oxygen and nitrogen species (ROS and RNS, respectively) scavenging capacity, phytochemical profile, and *in vitro* intestinal effects.

2. Materials and methods

2.1. Materials

All reagents used in the present work were from Sigma-Aldrich (Steinheim, Germany), Sigma Chemical Co. (St. Louis, USA), Merck (Darmstadt, Germany), Invitrogen Corporation (Life Technologies, S.A., Madrid, Spain) and Biowest (Nuaillé, France). The human colorectal adenocarcinoma (Caco-2; passages 9–10) and the human intestinal epithelial cells (HT29-MTX; passages 47–52) were from the American Type Culture Collection (ATCC, Manassas, VA, USA).

2.2. Samples

Dried *L barbarum* berries were purchased from Naturefoods, in October 2022, at a local store located in Porto, Portugal. The berries were crushed using a miller (Moulinex A320, France) and stored in a dark place until further analysis.

2.3. Ultrasound-assisted extraction

UAE was executed using an ultrasonic probe processor (Sonic Vibracell, model VCX50, Newtown, CT, USA) equipped with a probe tip No. 630–0219, featuring a 13 mm diameter and a frequency of 20 KHz. The experiments were carried out considering the RSM design detailed in Section 2.4., using water as solvent. After extraction, samples were centrifuged at 5000 rpm (Megafuge™ 16, Thermo Scientific, Massachusetts, USA) for 45 min at 20 °C for debris removal and filtered through Whatman n °1 paper. Then, the samples were frozen at −80 °C to further lyophilization (Cryodos–80, Telstar, Barcelona, Spain) and stored at 4 °C until further analysis.

2.4. Response surface methodology

RSM was employed to raise the extraction of bioactive compounds from goji berries using UAE and enhance the total phenolic content (TPC), as well as antioxidant and antiradical activities. A Box-Behnken design was chosen for this purpose. The independent variables analyzed included solid: liquid ratio (X_1), time (X_2) and intensity (X_3) in three different ratios (2.5, 6.25 and 10 %, *w/v*), time (20, 40 and 60 min) and intensity (30, 50 and 70 W/m^2). A total of 17 experiments were randomly performed, with 5 center points (X_1: 6.25 %; X_2: 40 min; X_3: 50 W/m^2). The dependent variables assessed in the experimental designs were TPC (Y_1, mg of gallic acid equivalents (GAE)/g of extract on dry weight (dw)), and antioxidant/antiradical activities evaluated by ABTS (Y_2, mg of ascorbic acid equivalents (AAE)/g of extract on dw), DPPH (Y_3, mg of trolox equivalents (TE)/g of extract on dw) and FRAP (Y_4, μmol of ferrous sulphate equivalents (FSE)/g dw) assays. The results were subjected to statistical analysis using the Design Expert Version 13 (Stat-Ease Inc., Minneapolis, MN, USA) to predict the model fitting. Moreover, this analysis enabled the determination of the optimal extraction conditions based on the desirability function, as well as the response surfaces, contour plots, and predictive regression equations. A denoting significance of $p < 0.05$ was accepted. Subsequently, a new set of experiments was carried out under the optimal conditions predicted by the RSM model, and the experimental values obtained were compared with those predicted by the model using a *t*-test.

2.4.1. Total phenolic content

The Folin–Ciocalteu method was used to determine the total phenolic content (TPC) (Singleton & Rossi, 1965). Gallic acid was used as standard curve (linearity range = 5 – 100 μg/mL; $R^2 > 0.998$) and the results were expressed as mg of gallic acid equivalents (GAE) per gram of extract on dw (mg GAE/g dw).

2.4.2. Antioxidant/antiradical activities

DPPH (Barros, Baptista, & Ferreira, 2007) and ABTS (Re, Pellegrini, Proteggente, Pannala, Yang, & Rice-Evans, 1999) radical scavenging potential were performed using, respectively, Trolox (linearity range = 5 – 125 μg/mL; $R^2 > 0.995$) and ascorbic acid (linearity range = 5 – 100 μg/mL; $R^2 > 0.988$) as standards. The results were respectively expressed as mg of trolox equivalents (TE) per gram of extract on dw (mg TE/g dw) and mg of ascorbic acid equivalent (AAE) per gram of extract on dw (mg AAE/g dw). Ferric reducing antioxidant power (FRAP) was assessed (Benzie & Strain, 1996) using ferrous sulfate heptahydrate ($FeSO_4{\cdot}7H_2O$) as standard solution (linearity range = 25 – 500 μM; $R^2 > 0.990$). The results were expressed in μmol of ferrous sulphate equivalents (FSE) per gram of extract on dw (μmol FSE/g dw).

2.5. Characterization of the L. Barbarum berries extract

2.5.1. Phenolic profile of L. barbarum extract by LC/DAD-ESI-MS

Phytochemical profile of the optimal *L. barbarum* berries extract was determined using liquid chromatography coupled with triple quadruple mass spectrometry (LC/DAD-ESI-MS), following the procedure described by Silva et al. (2022) (Silva, Almeida, et al., 2022). The stationary phase employed was an Agilent Eclipse XDB C-18 (3.0 × 150 mm) 3.5 μm column. The mobile phase consisted of a gradient of three compounds: water 1 % formic (A), acetonitrile (B) and methanol (C). The gradient began at 95 % A, 5 % B and 0 % C and continued to 0 % A, 90 % B and 10 % C, over 30 min. The flow rate was set at 0.4 mL/min, and the column temperature maintained at 30 °C. The sample was diluted 10 times in methanol: water (50:50, *v/v*) and centrifuged at 13300 rpm for 15 min. MS spectra were recorded in negative ion mode within a *m/z* range of 150——2000. For the quantification, reference compounds were selected based on available reference standard and identified compounds. Chlorogenic acid was used as reference compound for all phenylpropanoid derivatives, rutin was used as reference compound for all flavonoids and corosolic acid was used as reference compound for all flavonoids. For all nitrogen containing compounds ascribable to Lyciumspermidine there are not commercially available reference compounds, therefore chlorogenic acid was the external reference compound due to the presence of phenyl moiety, in order to assess semiquantitative measurement. For the analyzed compounds the following standard calibration curves were obtained and the compounds were semiquantified on the basis of the reference compound sharing more similar chemical structure:

Chlorogenic acid curve: $y = 198.01x + 20.138$ ($R^2 = 1$)
Rutin curve: $y = 58.564x + 41.752$ ($R^2 = 0.9998$)
Corsolic acid curve: $y = 8.52\ x\ + 0.25$ ($R^2 = 0.9989$)

The results obtained were expressed as mg of each compound per 100 g of dried sample (mg compound/100 g dw).

2.5.2. Reactive oxygen and nitrogen species scavenging capacity

2.5.2.1. Superoxide radical scavenging assay. The $O_2^{\bullet-}$ scavenging capacity was evaluated. (Gomes et al., 2007). The reaction mixture was composed of β-Nicotinamide adenine dinucleotide (NADH) (166 μM), nitrotetrazolium blue chloride (NBT) (43 μM), phenazine methosulfate (PMS) (2.7 μM) and sample (in a range of concentrations between 31.25 μg/mL and 1000 μg/mL). Gallic acid and catechin were used as positive controls and the assay were performed at room temperature. The absorbance was read at 560 nm for 6 min at 37 °C, and the results were expressed as the inhibition (IC_{50}) of NBT reduction to diformazan.

2.5.2.2. Hypochlorous acid scavenging assay. In what concerns to hypochlorous acid (HOCl) radical scavenging assay, the determination of HOCl concentration was performed spectrophotometrically, at a wavelength of 235 nm (ε 100 M^{-1} cm^{-1}) (Gomes et al., 2007). This methodology was carried out by mixing phosphate buffer (100 mM), sample (in a range of concentrations between 3.91 μg/mL and 125 μg/mL), dihydrorhodamine 123 (DHR) (5 μM) and HOCl (5 μM). Fluorescence was read at a wavelength of 485 ± 20 nm and 528 ± 20 nm at 37 °C for 5 min. Finally, IC_{50} was calculated to express the results in that order of magnitude.

2.5.2.3. Peroxyl radical scavenging assay. The $ROO^{\bullet}$ was generated by thermos decomposition of 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH) at 37 °C and pH 7.4 (Gomes et al., 2007). In each well, sample (in a range of concentrations between 1.95 μg/mL and 62.50 μg/mL), fluorescein (61.2 nM) and AAPH (19.1 mM) were mixed. The samples were previously prepared in 75 mM phosphate buffer with a pH of 7.4. The microplate was then incubated for 2 h in a microplate reader where the fluorescence intensity was measured at each minute at the emission wavelength 485 ± 20 nm with excitation at 528 ± 20 nm until total decay of florescence. Gallic acid (0.0469 to 1.5 μg/mL) and catechin (0.0625 to 0.2 μg/mL) were used as positive controls and Trolox (0.469 to 1.5 μg/mL) as a standard. The results were expressed as micromole of trolox equivalents per milligram of extract on dw (μmol TE/mg dw).

2.5.2.4. Hydrogen peroxide scavenging assay. The H_2O_2 scavenging capacity was determined by a chemiluminescent methodology (Gomes et al., 2007). The reaction mixture was prepared by mixing Tris buffer (50 mM) (with a pH of 7.4) and sample at different concentrations. Afterwards, lucigenin (800 μM) and 30 % H_2O_2 were added to the mixture. Gallic acid and catechin (1000 μg/mL) were used as positive controls. The chemiluminescent signal was detected immediately after the reaction mixture was complete in 5 min. The results were expressed as percentage of inhibition.

2.5.2.5. Peroxynitrite in the absence of $NaHCO_3$ scavenging assay. Regarding the scavenging capacity of $ONOO^-$ (Gomes et al., 2007), the determination of peroxinitryte concentration was performed spectrophotometrically at a wavelength of 302 nm (ε 1670 M^{-1} cm^{-1}). This methodology was carried out by mixing DHR (7.5 μM), sample (in a range of concentrations between 31.25 μg/mL and 1000 μg/mL) and $ONOO^-$ (600 nM). Fluorescence was read at a wavelength of 485 ± 20 nm and 528 ± 20 nm at 37 °C for 5 min. Finally, the IC_{50} was calculated to express the results in the percentage of $ONOO^-$-induced oxidation of DHR when in the absence of $NaHCO_3$.

2.5.2.6. Peroxynitrite in the presence of $NaHCO_3$ scavenging assay. The scavenging capacity of $ONOO^-$ was also assessed in the presence of $NaHCO_3$ (Gomes et al., 2007). The determination of peroxinitryte concentration was performed spectrophotometrically at a wavelength of 302 nm (ε 1670 M^{-1} cm^{-1}). This methodology was carried out by mixing DHR (7.5 μM), $NaHCO_3$ (187.5 mM), sample (in a range of concentrations between 31.25 μg/mL and 1000 μg/mL) and $ONOO^-$ (600 nM). Fluorescence was read at a wavelength of 485 ± 20 nm and 528 ± 20 nm at 37 °C for 5 min. The IC_{50} was calculated to express the results in the percentage of $ONOO^-$-induced oxidation of DHR when in the presence of $NaHCO_3$.

2.6. Cell viability assays

Caco-2 (cell line of human colorectal adenocarcinoma) and HT29-MTX (intestinal epithelial cell) were used to perform the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Silva, Almeida, et al., 2022). Briefly, cell lines were seeded at a concentration of 5 × 10^4 cells/mL and incubated with different concentrations of the optimal goji berries extract (0.1 – 1000 μg/mL), for 24 h. After that, MTT added for 3 h and DMSO was used to solubilize the crystals. Absorbance reading was performed at 570 nm and 690 nm to obtain a background. Positive (DMEM) and negative (Triton X-100 1 %, *w/v*) controls were used. The results obtained were expressed in percentage of cell viability.

2.7. Co-culture intestinal permeability model

The intestinal permeability was assessed resorting a co-culture model composed by 2 intestinal cell lines, namely Caco-2 and HT29-MTX, in a proportion of 90:10 (Silva, Almeida, et al., 2022). The assay was performed in a 12-well Transwell™ plate (well diameter of 22.1 mm, 3.8 cm^2) and Caco-2 and HT29-MTX cells were seeded on the apical chamber of Transwell™ inserts (insert diameter of 11.9 mm, 1.1 cm^2), with a final density of 1.1 × 10^5 cells/cm^2 in each insert. Cells were maintained in the same conditions for 14 days with medium changes every other day. After that, until the 21st day of the assay, only the apical medium was changed every other day. At the 21st days of assay,

goji berries extract (500 μg/mL) was added to the apical side of the co-culture layers. At different timepoints (15, 30, 45, 60, 90, 120, 180 and 240 min), and with HBSS replacement, samples (0.2 mL) from the basolateral side were collected. The Transepithelial Electrical Resistance (TEER) of the model was assessed before, during and at the end of the permeability assay through an EVOM Epithelial Volthometer Instrument equipped with a chopstick electrode (World Precision Instruments, Sarasota, FL, USA). The permeability results were expressed as relative percentage of transport.

2.7.1. Quantification of bioactive compounds permeation by LC/DAD-ESI-MS

The compounds from the optimal goji berries extract that potentially crossed the co-culture intestinal model were determined by LC/DAD-ESI-MS, following the procedure described in section 2.5.1. The results were expressed as permeation percentage (%) calculated by the ratio of the mass of each compound that permeated the cell layer at each time-point and the mass of each compound present in the optimal extract that was initially added in the apical side of the co-culture layer.

2.8. Statistical analysis

All measurements were performed in triplicate ($n = 3$) and the results were expressed as mean ± standard deviation (SD). A value of $p < 0.05$ was considered significant after using a one-way analysis of variance (ANOVA) followed by Tukey's HSD test, through the IBM SPSS Statistics 27.0 software (SPSS Inc., Chicago, IL, USA). Design Expert version 13 (Stat-Ease Inc, Minneapolis, MN, USA) allowed the obtention of the response surface analysis, points of prediction of the variables under study and the statistical analysis of the RSM design.

3. Results and discussion

3.1. Validation of the RSM model

RSM is a powerful mathematical tool to explore the impact of dependent variables on independent ones (Prodromos Skenderidis et al., 2017). In the present work, RSM was applied to optimize the UAE extraction of goji berries using solid:liquid ratio (%, *w/v*), ultrasonic time (min) and intensity (W/m^2) as independent variables, while TPC (mg GAE/g) and antioxidant/antiradical activities (ABTS (mg AAE/g), DPPH (mg TE/g) and FRAP (μmol FSE/g)) were the dependent ones. The predicted and experimental values of TPC, ABTS, DPPH and FRAP assays for all points predicted by the RSM software are summarized in Table 1.

According to the obtained results, the TPC ranged between 17.75 mg GAE/g dw (run 16: X_1: 10 %; X_2: 40 min; X_3: 30 W/m^2) and 20.91 mg GAE/g dw (run 1: X_1: 10 %; X_2: 60 min; X_3: 50 W/m^2), values in line with studies conducted by other authors. For instance, Mocan et al. (2018) reported a TPC of 15.70 mg GAE/g dw for goji berries extracted by a sonication bath with methanol/water (70:30, *v/v*) for one hour (Mocan et al., 2018), using a solid:liquid ratio of 10 % (*w/v*). Moreover, a TPC of 14.118 mg GAE/g dw was achieved for goji berries extracted by ultrasound bath at 65 °C for 1 h, employing 75 % of ethanol and a solid:liquid ratio of 1:10 (*w/v*) (Feng et al., 2021).

Table 1
Experimental and predicted values of TPC, ABTS, DPPH and FRAP for all prepared extracts.

Point	Independent Variables Extraction Conditions			Dependent Variables Y_1, TPC (mg GAE/g dw)		Y_2, ABTS (mg AAE/g dw)		Y_3, DPPH (mg TE/g dw)		Y_4, FRAP (μmol FSE/g dw)	
Run	X_1 (solid:liquid ratio, % *w/v*)	X_2 (time, min)	X_3 (intensity, W/m^2)	Exp *	Pred	Exp *	Pred	Exp *	Pred	Exp *	Pred
1	10	60	50	20.91 ± 1.36	20.72	14.56 ± 1.20	13.91	7.85 ± 0.80	7.12	106.00 ± 10.07	99.88
2	6.25	60	70	20.52 ± 0.49	20.52	10.09 ± 0.69	11.04	7.09 ± 0.35	7.98	95.00 ± 8.86	98.15
3	10	20	50	19.81 ± 1.22	19.68	10.50 ± 1.96	11.24	3.43 ± 0.21	3.88	73.85 ± 17.68	75.65
4	6.25	40	50	19.16 ± 1.19	19.17	14.95 ± 0.95	13.85	9.84 ± 1.00	9.07	106.79 ± 11.25	105.24
5	6.25	40	50	19.01 ± 1.15	19.17	14.29 ± 0.84	13.85	10.29 ± 1.10	9.07	105.09 ± 9.33	105.24
6	6.25	60	30	18.91 ± 0.69	18.98	9.93 ± 1.16	10.31	5.80 ± 0.37	6.08	83.28 ± 2.35	88.05
7	6.25	40	50	19.24 ± 1.39	19.17	13.55 ± 1.09	13.85	9.25 ± 0.54	9.07	104.17 ± 7.98	105.24
8	2.5	60	50	20.18 ± 1.42	20.31	10.00 ± 0.42	9.33	6.09 ± 0.45	5.64	83.86 ± 6.23	82.06
9	2.5	40	70	18.68 ± 1.71	18.56	12.95 ± 0.96	12.68	8.24 ± 1.63	7.79	85.00 ± 8.72	83.65
10	6.25	40	50	19.04 ± 1.15	19.17	14.16 ± 0.96	13.85	7.89 ± 0.29	9.07	104.30 ± 7.30	105.24
11	2.5	20	50	20.15 ± 0.62	20.34	10.20 ± 1.13	10.85	7.06 ± 0.58	7.79	88.70 ± 2.89	94.82
12	10	40	70	18.97 ± 0.83	19.17	14.55 ± 1.20	14.26	6.96 ± 0.67	6.80	97.00 ± 10.39	99.98
13	2.5	40	30	18.94 ± 0.65	18.74	9.13 ± 1.03	9.42	5.60 ± 0.59	5.76	90.00 ± 10.92	87.03
14	6.25	20	70	19.10 ± 1.65	19.03	12.46 ± 1.54	12.08	8.08 ± 0.81	7.79	100.72 ± 11.23	95.95
15	6.25	40	50	19.40 ± 1.12	19.17	12.30 ± 0.88	13.85	8.10 ± 0.55	9.07	105.83 ± 6.49	105.24
16	10	40	30	17.75 ± 1.36	17.88	12.54 ± 1.12	12.81	3.87 ± 0.43	4.32	68.00 ± 5.84	69.35
17	10	60	50	19.46 ± 0.97	19.46	9.06 ± 0.33	8.12	6.08 ± 0.55	5.18	81.93 ± 8.16	78.78

TPC – Total phenolic content; ABTS – ABTS radical scavenging assay; DPPH – DPPH radical scavenging assay; FRAP – Ferric-reducing antioxidant power; Exp – Experimental; Pred – Predicted. * Results are expressed as mean ± standard deviation ($n = 3$).

Regarding ABTS, the values varied from 9.06 mg AAE/g dw (run 17: X_1: 10 %; X_2: 60 min; X_3: 50 W/m^2) to 14.95 mg AAE/g dw (run 4: X_1: 6.25 %; X_2: 40 min; X_3: 50 W/m^2). Mendes et al. (2016) also evaluated the ABTS scavenging capacity of *L. barbarum* berries extracted by Microwave-Assisted Extraction (MAE) at 100 °C for 8 min, using 25 % of methanol as solvent, and reported values between 5.5 and 7.6 mg AAE/g dw (Mendes et al., 2016), values lower than the ones achieved in this study.

In what concerns to DPPH, the results ranged between 3.43 mg TE/g dw (run 3: X_1: 10 %; X_2: 20 min; X_3: 50 W/m^2) and 10.29 mg TE/g dw (run 5: X_1: 6.25 %; X_2: 40 min; X_3: 50 W/m^2), being in accordance with the ones reported for a extract obtained by sonication bath for 1 h at room temperature and using methanol/water (70:30, *v/v*) as solvent (9.35 mg TE/g dw) (Mocan et al., 2018). Oppositely, Feng et al. (2021) described a DPPH scavenging capacity of 36.80 mg TE/g dw for a goji berries extract obtained by ultrasound bath with 75 % of ethanol and a solid:liquid ratio of 1:10 (*w/v*), performed at 65 °C for 1 h (Feng et al., 2021).

The FRAP results ranged between 68 μmol FSE/g dw (run 16: X_1: 10 %; X_2: 40 min; X_3: 30 W/m^2) and 106.79 μmol FSE/g dw (run 4: X_1: 6.25 %; X_2: 40 min; X_3: 50 W/m^2), being higher than the values achieved by Benchennouf et al. (2017). The authors reported values of 80.00 μmol FSE/g dw, being the extract prepared by a conventional solid–liquid extraction with water and orbital shaking of the extraction solution for 3 h at 300 rpm (Benchennouf, Grigorakis, Loupassaki, & Kokkalou, 2017).

Table 2 summarizes the adequacy and significance of the obtained values by the RSM, according to the ANOVA test analysis that allowed the application of the quadratic model. The following parameters must be confirmed to ensure the veracity of the model: adequate precision higher than 4, significant *p*-value (<0.05) and R^2 value close to 1.

The adequate precision was higher than 4 in all responses (Y_1, Y_2, Y_3 and Y_4), supporting an adequate model fitting and signal-to-noise ratio. TPC (Y_1) showed a high R^2 value and adjusted R^2 (0.9644 and 0.9186, respectively), similarly to FRAP (Y_4, 0.9275 and 0.8343, respectively), reenforcing the adequacy of the model to the dependent variables. Besides the low adjusted R^2 values obtained for the ABTS (Y_2) and DPPH (Y_3) response, based on the non-significance ($p > 0.05$) of the lack of fit and the adequate precision above 4 for this response, it was possible to successfully validated the experimental model.

Regarding the independent variables (X_1, X_2 and X_3), it is possible to observe that X_1 had a significant effect ($p = 0.0139$) on ABTS (Y_2), while X_2 significantly affected ($p = 0.0137$) TPC (Y_1). In what concerns to X_3, it significantly affected ($p < 0.05$) all responses (Y_1, Y_2, Y_3 and Y_4). The "lack of fit" values did not present significant effects ($p > 0.05$) on any response, except Y_4 ($p = 0.0014$). The R^2 value (0.9275) of this response validates the experimental model, as well as the adequate precision (9.4147) and the significance of the module ($p = 0.0031$). Also, it is relevant to highlight that all the variables evaluated (Y_1, Y_2, Y_3 and Y_4) were significant ($p < 0.05$) for the model applied, reinforcing the validation and effectiveness of the experimental design.

The following quadratic equations (Equations (1) to (4)), in terms of codded factors, exhibit the relation between the dependent variables, namely TPC (Y_1), ABTS(Y_2), DPPH (Y_3) and FRAP (Y_3), and the independent ones, namely solid:liquid ratio (X_1), time (X_2) and intensity (X_3). The equations allow the prediction of the response of each variable.

$$Y_1 = 19.17 - 0.0638X_1 + 0.2500X_2 + 0.2762X_3 + 0.2675X_1X_2 + 0.3700X_1X_3 + 0.4925X_2X_3 + 0.0900X_1^2 + 1.00X_2^2 - 0.6750X_3^2 \quad (1)$$

$$Y_2 = 13.85 - 1.24X_1 + 0.2862X_2 + 1.17X_3 + 1.05X_1X_2 - 0.4525X_1X_3 - 0.8100X_2X_3 - 0.3050X_1^2 - 2.21X_2^2 - 1.25X_3^2 \quad (2)$$

Table 2
RSM model analysis through ANOVA statistical test of TPC, ABTS, DPPH and FRAP of the prepared extracts.

Source	Sum of squares				Mean squares				F value				*p*-value			
	Y_1	Y_2	Y_3	Y_4	Y_1	Y_2	Y_3	Y_4	Y_1	Y_2	Y_3	Y_4	Y_1	Y_2	Y_3	Y_4
Model	8.86	61.43	48.50	2212.73	0.9848	6.83	5.39	245.86	21.06	5.87	4.63	9.95	0.0003	0.0146	0.0278	0.0031
X_1	0.0325	12.35	2.98	0.9180	0.0325	12.35	2.98	0.9180	0.6952	10.63	2.56	0.0372	0.4319	0.0139	0.1536	0.8526
X_2	0.5000	0.6555	0.5940	65.78	0.5000	0.6555	0.5940	65.78	10.69	0.5640	0.5110	2.66	0.0137	0.4771	0.4979	0.1467
X_3	0.6105	11.02	10.17	371.42	0.6105	11.02	10.17	371.42	13.05	9.48	8.75	15.04	0.0086	0.0178	0.0212	0.0061
X_1, X_2	0.2862	4.39	7.26	342.07	0.2862	4.39	7.26	342.07	6.12	3.78	6.25	13.85	0.0426	0.0931	0.0410	0.0074
X_1, X_3	0.5476	0.8190	0.0506	289.00	0.5476	0.8190	0.0506	289.00	11.71	0.7047	0.0435	11.70	0.0111 *	0.4289	0.8406	0.0111 *
X_2, X_3	0.9702	2.62	0.1260	12.50	0.9702	2.62	0.1260	12.50	20.75	2.26	0.1084	0.5059	0.0026	0.1766	0.7516	0.4999
X_1^2	0.0341	0.3917	13.35	526.57	0.0341	0.3917	13.35	526.57	0.7292	0.3370	11.48	21.32	0.4214	0.5797	0.0116	0.0024
X_2^2	4.23	20.61	5.92	149.09	4.23	20.61	5.92	149.09	90.48	17.74	5.09	6.04	< 0.0001	0.0040	0.0586	0.0437
X_3^2	1.92	6.61	5.34	345.08	1.92	6.61	5.34	345.08	41.02	5.68	4.59	13.97	0.0004	0.0486	0.0694	0.0073
Residual	0.3274	8.14	8.14	172.92	0.0468	1.16	1.16	24.70								
Lack of fit	0.2270	4.14	3.69	168.12	0.0757	1.38	1.23	56.04	3.01	1.38	1.11	46.68	0.1571	0.3691	0.4441	0.0014
Pure error	0.1004	3.99	4.45	4.80	0.0251	0.9980	1.11	1.20								
Total	9.19	69.57	56.64		2385.64											
R^2 (Y_1) = 0.9644					R^2 pred (Y_1) = 0.5878					R^2 adjust (Y_1) = 0.9186				Adeq Precision (Y_1) = 17.1301		
R^2 (Y_2) = 0.8831					R^2 pred (Y_2) = -0.0425					R^2 adjust (Y_2) = 0.7327				Adeq Precision (Y_2) = 7.4276		
R^2 (Y_3) = 0.8563					R^2 pred (Y_3) = -0.1655					R^2 adjust (Y_3) = 0.6716				Adeq Precision (Y_3) = 6.2837		
R^2 (Y_4) = 0.9275					R^2 pred (Y_4) = -0.1307					R^2 adjust (Y_4) = 0.8343				Adeq Precision (Y_4) = 9.4147		

Y_1 – TPC (mg GAE/g dw); Y_2 – ABTS (mg AAE/g dw); Y_3 – DPPH (mg TE/g dw); Y_4 – FRAP (μmol FSE/g dw); X_1 – solid:liquid ratio (%w/v); X_2 – time (min); X_3 – intensity (W/m^2).

$$Y_3 = 9.07 - 0.6100X_1 + 0.2725X_2 + 1.13X_3 + 1.35X_1X_2 + 0.1125X_1X_3 - 0.1775X_2X_3 - 1.78X_1^2 - 1.19X_2^2 - 1.13X_3^2 \quad (3)$$

$$Y_4 = 105.24 - 0.3388X_1 + 2.87X_2 + 6.81X_3 + 9.25X_1X_2 + 8.50X_1X_3 - 1.77X_2X_3 - 11.18X_1^2 - 5.95X_2^2 - 9.05X_3^2 \quad (4)$$

3.2. Response surface analysis

The graphic relationship between dependent (TPC, ABTS, DPPH and FRAP assays) and independent variables (solid: liquid ratio, time, and intensity) is represented in the 3D graphs obtained by the RSM experimental design (Fig. 1). The intensity was fixed on 50 W/m^2.

According to Fig. 1, the TPC noticeably differed from the other responses determined by the ABTS, DPPH, and FRAP experiments. In fact, when the extraction time and the solid:liquid ratio varied between the maximum values assessed (40 and 60 min and 5 and 10 % (*w/v*), respectively), a stronger antioxidant (FRAP = 106.79 mol FSE/g dw) and antiradical (ABTS = 14.95 mg AAE/g dw; DPPH = 10.29 mg TE/g dw) activities were observed. This data suggests that under the optimal UAE conditions more bioactive compounds are extracted from the natural matrix. This hypothesis is reaffirmed by the TPC achieved (TPC = 20.91 mg GAE/g dw) when the maximum time and solid:liquid ratio (60 min and 10 % (*w/v*), respectively) were employed (Fig. 1 (I)). In general, higher extraction times (40 – 60 min) and solid:liquid ratios (6.25 – 10 % (*w/v*)) led to better responses, which is corroborated by the desirability graph (Fig. 2). In fact, higher ultrasonic times promote indirect heating of the extraction solution, leading to an enhanced solubility of specific compounds and, consequently, higher extraction of phenolic compounds. Regarding intensity, it is noteworthy that its impact was negligible on all responses studied. Still, it is anticipated that the employment of higher sonication intensities will result in the extraction of greater amounts of compounds since generates an increase in the formation of cavitation bubbles and elevated the pressure, leading to an extremely efficient cell rupture and extraction of intracellular compounds (Prodromos Skenderidis et al., 2017).

The desirability graph (Fig. 2) shows the predicted optimal extraction conditions that allow to maximize the response variables (TPC, ABTS, DPPH and FRAP). The predicted optimal UAE conditions are a

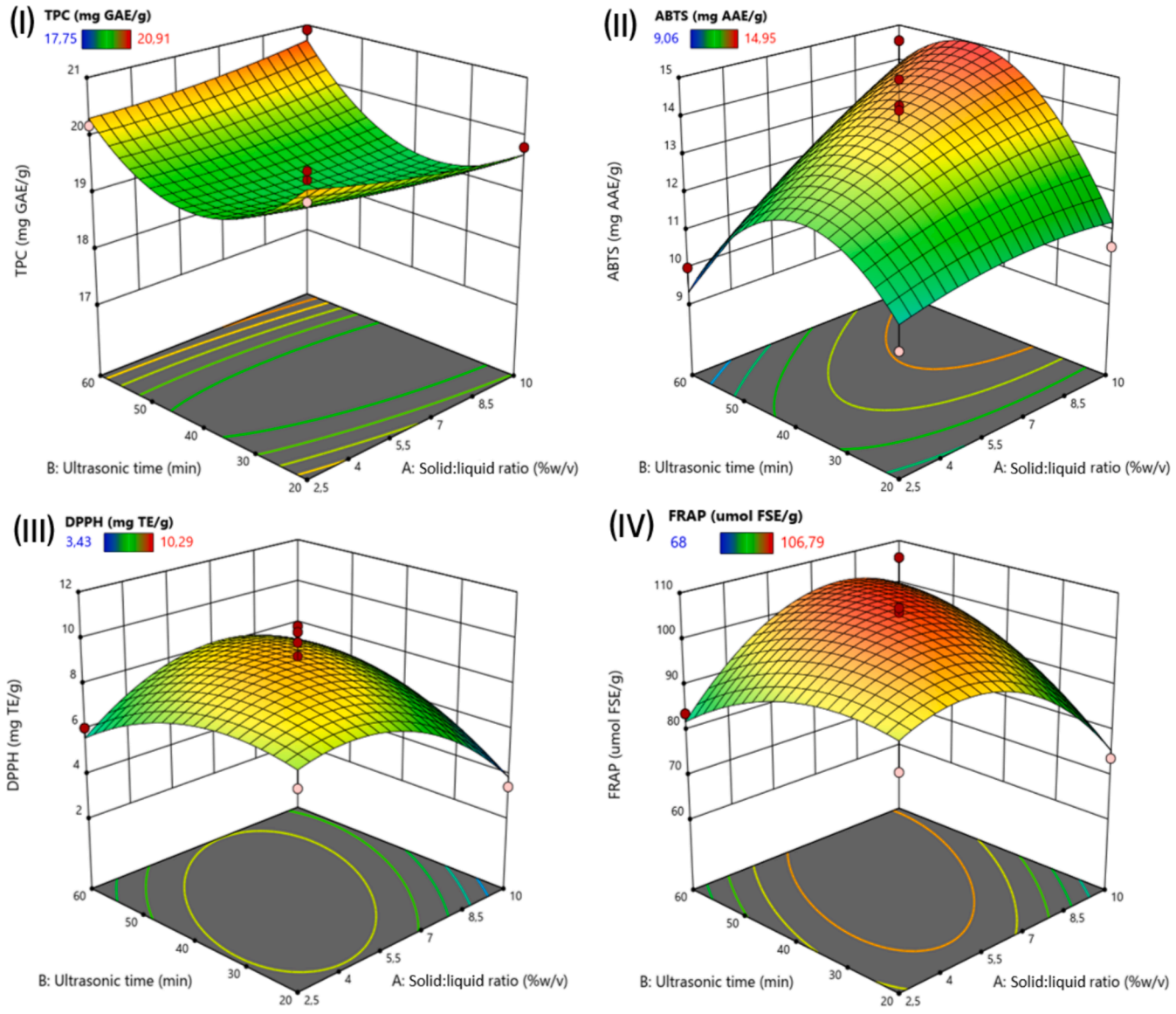

Fig. 1. RSM 3D graphics for the interaction between independent (solid:liquid ratio and ultrasonic time) and dependent variables (I: TPC, II: ABTS, III: DPPH, IV: FRAP).

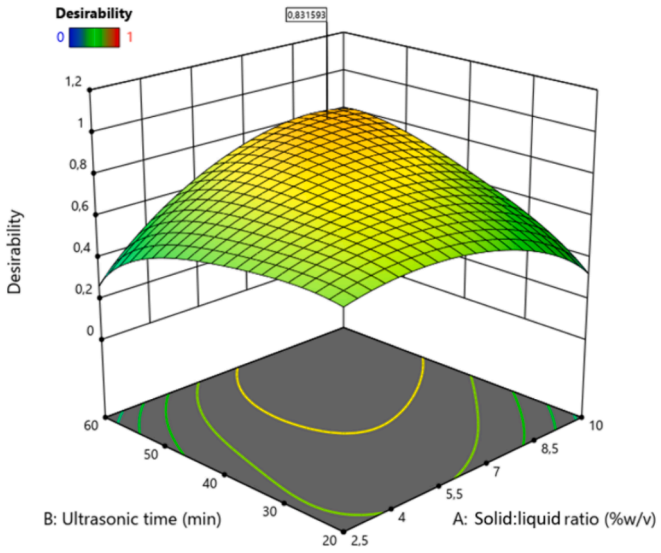

Fig. 2. RSM 3D graphics for the desirability index.

Table 3
Experimental and predicted value of TPC, ABTS, DPPH and FRAP for optimal goji berries extract.

	TPC (mg GAE/g)	ABTS (mg AAE/g)	DPPH (mg TE/g)	FRAP (μmol FSE/g)
Experimental value*	23.87 ± 1.47	15.15 ± 1.13	10.25 ± 0.81	105.97 ± 8.20
Predicted value	20.45	13.73	8.29	106.60
p-value	0.066	0.067	0.258	0.266

* Results are expressed as mean ± standard deviation ($n = 3$).

solid:liquid ratio of 8.75 %, for 56.21 min through an ultrasonic intensity of 59.05 W/m^2 ($R^2 = 0.831593$). After performing the UAE under optimal conditions, the dependent variables (TPC, ABTS, DPPH and FRAP) were evaluated and no significant differences ($p > 0.05$) were observed between the predicted values and the experimental ones (Table 3), validating the RSM model used in this work.

3.3. Characterization of the optimal *L. barbarum* berries extract

3.3.1. Phytochemical profile

Table 4 summarizes the compounds identified in the optimal goji berries extract by LC/DAD-ESI-MS based on the retention time and the fragment ions detected by the mass spectrometer (Supplementary Fig. 1).

It is well-known that cultivation factors, particularly variety, geographic location, soil, and climatic conditions, play a crucial role in the definition of the phytochemical profile of a plant (Zhao et al., 2022). Depending on these intrinsic and extrinsic factors, the plants metabolism produces different bioactive compounds (D. Almeida et al., 2018). Moreover, the extraction techniques and conditions employed, especially the solvent used, impact the extracts phytochemical profile (Jiang, Fang, Leonard, & Zhang, 2021).

As can be observed in Table 4, a total of fifteen phytochemical compounds were identified in the optimal goji berries extract: two phenolic acids (5.96 mg/100 g dw), five flavonoids (36.01 mg/100 g dw), and eight other compounds (375.97 mg/100 g dw). Among these categories, flavonoids (peaks 8, 11, 12, 13 and 14) were the most abundant phenolic compounds (36.01 mg/100 g dw), with rutin (19.27 mg/100 g dw) being the principal one, followed by derivatives, such as rutin hexoside (1.39 mg/100 g dw) and rutin exoside (12.85 mg/100 g dw). The second category of phenolic compounds detected in highest amounts (5.96 mg/100 g dw) were phenolic acids (peaks 1 and 2), comprising feruloylquinic acid (0.20 mg/100 g dw) and 3,5-dicaffeoylquinic acid (5.76 mg/100 g dw). These phenolic compounds hold significant potential for therapeutic administration (Jiang et al., 2021). For instance, rutin has a strong antioxidant potential, acting as a powerful free-radical scavenger (Vasantha Rupasinghe et al., 2014). This holds true for its several derivatives as well, including rutin hexoside, rutin exoside, kaempferol-3-*O*-rutinoside, and isorhamnetin-3-*O*-rutinoside (Wu, Lv, Wang, & Wang, 2016).

Similarly to the present work, different authors have identified some phenolic compounds in *L. barbarum* berries. Xiao et al. (2019) reported the presence of rutin, spermidine, 3-glu-kukoamine, 2-glu-kukoamine, glu-lycibarbarspermidine F and lycibarbarspermidine B in goji berries extract prepared in an ultrasound bath using ethanol and water (70:30, *v/v*) as solvent (Xiao et al., 2019). Various glycosylated kukoamines, lycibarbarspermidines and glycosylated lycibarbarspermidines were also detected in other studies using other extraction techniques (dos Santos, de Almeida Veiga, Carlotto, Mello, Serrato, & de Souza, 2022).

Table 4
List of phytochemical compounds identified and quantified in the optimal *L. barbarum* berries extract through LC/DAD-ESI-MS analysis.

Peak	Compound	Rt (min)	$[M-H]^-$ (m/z)	Fragments (m/z)	Quantification (mg/100 g dw) *
	Phenolic acids				
1	Feruloylquinic acid	1.23	377	377, 191	0.20 ± 0.01
2	3,5-dicaffeoylquinic acid	3.46	515	515, 353, 191	5.76 ± 0.29
	∑ Phenolic acids				5.96 ± 0.30
	Flavonoids				
11	Rutin hexoside	7.09	771	771, 609, 301, 179, 151	1.39 ± 0.07
12	Rutin	8.20	609	609, 301, 179, 151	19.27 ± 0.96
13	Kaempferol-3-*O*-rutinoside	10.65	593	593, 285, 257, 229	0.90 ± 0.04
14	Isorhamnetin-3-*O*-rutinoside	11.52	623	623, 316, 300, 271	1.60 ± 0.08
8	Rutin exoside	4.51	771	771, 609, 301, 179, 151	12.85 ± 0.64
	∑ Flavonoids				36.01 ± 1.80
	Others				
3	3-Glu-kukoamine	3.54	1015	1015, 853, 690, 529	17.63 ± 0.88
4	2-Glu-kukoamine (isomer 1)	3.62	853	690, 529	10.22 ± 0.51
5	2-Glu-kukoamine (isomer 2)	3.79	853	853, 690, 529	104.96 ± 5.25
6	Glu-lycibarbarspermidine F (isomer 1)	4.15	956	956, 795, 470	15.18 ± 0.76
7	Glu-lycibarbarspermidine F (isomer 2)	4.50	956	956, 795, 470	126.72 ± 6.34
9	Lycibarbarspermidine B	4.80	632	632, 470, 334	17.07 ± 0.85
10	Spermidine	5.53	470		0.96 ± 0.05
15	Corosolic acid	22.60	471	471, 453	83.23 ± 4.16
	∑ Others				375.97 ± 18.80

Rt – Retention time. * Results are expressed as mean ± standard deviation ($n = 3$).

These compounds also have valuable biological activities (dos Santos et al., 2022). Corosolic acid, also known as plant insulin, is well known for its antidiabetic properties (Qian et al., 2021). The remaining compounds may fall under the category of phenylamides, often referred as hydroxycinnamic acid amides, being generated when hydroxycinnamic acids are conjugated with amines (W. Wang, Snooks, & Sang, 2020). These compounds exhibit similar bioactivity to the unconjugated compounds, since retain the properties of their parent molecules (W. Wang et al., 2020). Spermidines, characteristic chemical markers of *Lycium* fruits (Mocan et al., 2018), show pro-healthy properties, such as anti-aging, antihypertension, antidiabetic, and immune modulation (dos Santos et al., 2022; W. Wang et al., 2020). Their derivatives, like lycibarbarspermidines and kukoamines, demonstrated similar activities (W. Wang et al., 2020). For instance, lycibarbarspermidines present a potent antioxidant potential as well as the ability to improve the short-term memory in Alzheimer's patients (dos Santos et al., 2022). Kukoamines and their derivatives exhibit a wide range of bioactivities, including antihypertensive, antilipidic peroxidation and lipoxygenase effects, antisepsis and neuroprotection (Li, Wang, Zhao, Huang, Tang, & Cheung, 2015).

Xiao et al. (2019) analyzed a goji berries extract prepared by ultrasound bath and detected the presence of rutin by UPLC-Q-Orbitrap-MS/MS (Xiao et al., 2019) in lower quantities than the ones achieved in the present study (9.3 mg/ 100 g dw *vs* 19.27 mg/100 g dw − Table 4). The same occurred with Wu et al. (2016) that reported the presence of 3,5-dicaffeoylquinic acid (3.70 mg/100 g dw), kaempferol-3-*O*-rutinoside (0.30 mg/100 g dw) and isorhamnetin-3-*O*-rutinoside (0.70 mg/100 g dw) (Wu et al., 2016) in lower amounts than in the present study (5.76, 0.90 and 1.60 mg/100 g dw, respectively, Table 4) in an extract prepared in a ultrasound bath (Wu et al., 2016). All the studies mentioned above consistently report lower quantities of phenolic compounds than the present one. Given that none of them used the optimal UAE conditions established in the present study neither an ultrasonic probe, the data obtained allow to conclude that the optimal conditions predicted by the RSM model have a significant impact on the recovery of bioactive compounds from goji berries.

The overall presence of the phytochemical compounds detected in *L. barbarum* berries optimal extract play a vital role in the bioactivity of the extract, particularly regarding oxidative stress (dos Santos et al., 2022; Jiang et al., 2021). Hence, the phytochemical profile of the optimal extract is a preliminary indication that goji berries may hold a considerable bioactive composition with interest for nutraceutical or pharmaceutical industries.

3.3.2. *In vitro* scavenging capacity of ROS and RNS

ROS and RNS are physiologically generated in low concentrations, promoting important processes in organisms, such as homeostasis and cell signaling (D. Almeida et al., 2018; P. Skenderidis et al., 2018). However, if the concentration of these species exceeds the antioxidant/antiradical capacity of the cellular systems, a state of oxidative stress occurs (Vasantha Rupasinghe et al., 2014). Plants produce polyphenols as antioxidant/antiradical mechanisms to prevent the oxidative stress (Vasantha Rupasinghe et al., 2014) due to their capacity to neutralize reactive species and, therefore, prevent cell damage. Thus, it is important to assess the capacity of the optimal extract to scavenge ROS and RNS (Table 5).

$O_2^{\bullet -}$ and H_2O_2 play a crucial role in multiple physiological processes. While these species are not considered potent pro-oxidants, they are starting points for oxidative stress (I. F. Almeida, Fernandes, Lima, Costa, & Bahia, 2008) due to their capacity of acting as precursors of powerful pro-oxidants (D. Almeida et al., 2018). Regarding $O_2^{\bullet -}$, the optimal extract (IC_{50} = 225.25 μg/mL) demonstrated a significant lower ($p < 0.05$) quenching capacity than the positive controls employed, namely catechin and gallic acid (IC_{50} of 18.01 and 6.34 μg/mL, respectively). As for the H_2O_2 scavenging capacity of the optimal extract, it was not possible to determine the IC_{50} for the concentrations tested. Therefore, the extract H_2O_2 scavenging capacity was expressed in terms of percentages of inhibition for the maximum concentration tested (1000 μg/mL). The optimal extract achieved an inhibition of 16.60 %, while catechin showed an inhibition of 31.40 % and gallic acid reached an IC_{50} value of 193.50 μg/mL.

HOCl is a powerful oxidant physiologically produced by active neutrophils and monocytes as a defense mechanism against different threats (de Siqueira et al., 2022). The optimal extract exhibited a good scavenging capacity of HOCl (IC_{50} = 12.99 μg/mL), despite the positive controls achieved better results, namely IC_{50} of 0.20 μg/mL for catechin and 2.60 μg/mL for gallic acid.

Concerning the scavenging capacity of $ROO^{\bullet}$, it was not possible to calculate the IC_{50} at the maximum concentration of the goji berries extract tested (1000 μg/mL), being the results expressed as μmol TE/mg dw. The optimal extract demonstrated a good scavenging capacity (0.15 μmol TE/mg dw), while the positive controls, catechin and gallic acid, achieved scavenging capacities of 6.37 and 2.45 μmol TE/mg dw, respectively. These results are consistent with the previous studies that reported $ROO^{\bullet}$ scavenging capacities from 0.01 μmol TE/mg dw(Rocchetti et al., 2018) to 0.24 μmol TE/mg dw (Feng et al., 2021) for goji berries extracts prepared by different techniques (ultra-turrax and ultrasound bath, respectively) and under different extraction conditions (10 mL of 1 % formic acid in an 80:20 methanol/water solution at 25000 rpm for 3 min and 75 % ethanol with a solid:liquid ratio of 1:10 (*w/v*) at 65 °C for 1 h, respectively).

In what concerns to the scavenging capacity of RNS, specifically $ONOO^-$, a powerful pro-oxidant and detrimental compound (I. F. Almeida et al., 2008), the optimal goji berries extract achieved a noteworthy result in the absence of $NaHCO_3$, with an IC_{50} = 57.78 μg/mL. The same happen to the positive controls, catechin and gallic acid, accomplishing a better scavenging capacity in the absence of $NaHCO_3$ (IC_{50} = 0.19 and 0.20 μg/mL, respectively). It has previously been noted that the presence of $NaHCO_3$ allows a closer approximation to physiological conditions (Marangi et al., 2018). However, the presence of sodium bicarbonate may also lead to the formation of $ONOO^-$ derivatives, which affects the reactivity of $ONOO^-$ scavengers, possibly decreasing

Table 5
Superoxide anion radical ($O2^{\cdot -}$), hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl), peroxyl radical ($ROO^{\cdot}$), and peroxinitrite ($ONOO^{-}$) in the absence and presence of sodium bicarbonate ($NaHCO_3$) scavenging capacities of the optimized *L. barbarum* berries extract.

Sample	ROS				RNS	
	$O_2^{\bullet -}$ (IC_{50} (μg/mL))	H_2O_2	HOCl	$ROO^{\bullet}$ (μmol TE/mg dw)	$ONOO^-$ (IC_{50} (μg/mL))	$ONOO^-$ w/ $NaHCO_3$
Optimized extract	225.25 ± 53.26[b]	n.d.	12.99 ± 1.13[c]	0.15 ± 0.01[c]	57.78 ± 4.26[b]	93.51 ± 19.65[b]
Positive controls						
Catechin	18.01 ± 0.77 [a]	n.d.	0.20 ± 0.03 [a]	6.37 ± 2.32 [a]	0.19 ± 0.03 [a]	0.29 ± 0.07 [a]
Gallic acid	6.34 ± 0.53 [a]	193.50 ± 17.68	2.60 ± 0.14[b]	2.45 ± 0.54[b]	0.20 ± 0.004 [a]	0.28 ± 0.05 [a]

IC_{50} − *in vitro* concentration required to decrease in 50 % the reactivity of the studied reactive species; n.d. − IC_{50} was not determined up to the highest tested concentration (1000 μg/mL). Results are expressed as mean ± standard deviation ($n = 3$). Different letters ([a, b, c]) in the same column mean significant differences ($p < 0.05$).

their effects (Gomes et al., 2007; Marangi et al., 2018). This phenomenon has been verified in studies of other natural matrices such as *Actinidia arguta* leaves (Marangi et al., 2018), *Arrabidaea chica* leaves (de Siqueira et al., 2022), *Castanea sativa* and *Quercus robur* leaves (I. F. Almeida et al., 2008).

It is conceivable that the phenolic compounds quantified in the goji berries extract, such as feruloylquinic acid, 3,5-dicaffeoylquinic acid, rutin, and its derivatives, are responsible for the ROS and RNS scavenging activity observed, as attested by their capacity to neutralize ROS and RNS (Vasantha Rupasinghe et al., 2014). For instance, it has been attested in previous studies that the scavenging efficacy of flavonoids is positively influenced by an increased number of hydroxyl (OH) groups substitution in the C-ring of the phenolic structure (I. F. Almeida et al., 2008). Therefore, given the number and location of OH substitutions (Jiang et al., 2021; Silva, Almeida, et al., 2022), rutin and its derivatives may have a strong influence in the scavenging capacity of the optimal extract. Regarding phenolic acids, it has been established that the CH = CH-COOH group, which is present in the hydroxycinnamic acids, greatly contributes to the antioxidant efficiency of these compounds (Jiménez-Moreno et al., 2019). Therefore, given that phenolic compounds identified in the optimal goji berries extract are hydroxycinnamic acids, feruloylquinic acid and 3,5-dicaffeoylquinic acid, their contribution for the optimal extract free-radical scavenging potential should be considered. Nevertheless, one of the great advantages of natural plant extracts is the synergic effect of the different compounds present in their constitution (I. F. Almeida et al., 2008). Hence, the overall phytochemical compounds available in the optimal extract likely account for the demonstrated ROS and RNS scavenging capacity.

3.4. *In vitro cell studies*

The safety of the optimal goji berries extract was assessed by an MTT assay on intestinal cell lines, namely Caco-2 and HT29-MTX. These lines were selected as intestinal cell models to evaluate the potential effects of the optimal extract on small intestine, the principal place where the absorption of bioactive compounds occurs (Silva, Almeida, et al., 2022). The extract was tested in a concentration range between 0.1 and 1000 μg/mL and the results are presented in Fig. 3.

According to the obtained results, the optimal extract did not affect the viability of any cell lines in concentrations lower than 500 μg/mL, presenting viabilities between 80 % and 100 %. However, the highest tested concentration (1000 μg/mL) caused a significant decrease in the viability of both cell lines (60.00 % and 17.02 % in Caco-2 and HT29-MTX, respectively). Regarding Caco-2, the viability ranged between 60.00 % and 102.34 %, respectively for the concentration of 1000 μg/mL and 0.1 μg/mL, with significant differences ($p < 0.05$) between the highest concentration and the other concentrations tested. Concerning HT29-MTX, the viability varied between 17.02 % and 101.10 % for the concentration of 1000 μg/mL and 0.1 μg/mL, respectively. Still, the highest concentration tested (1000 μg/mL) led to a significant ($p < 0.05$) decrease in the HT29-MTX viability (17.02 %). Therefore, this study emphasizes the non-cytotoxic effect of the optimal goji berries extract in concentrations below 500 μg/mL in intestinal cell lines.

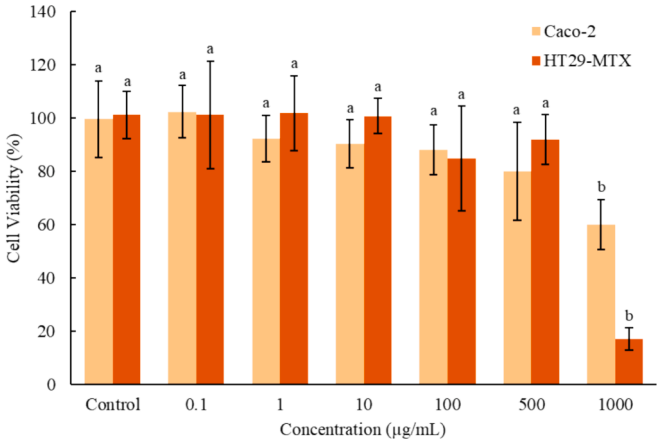

Fig. 3. Effects of the exposure of the optimal *L. barbarum* berries extract on Caco-2 and HT29-MTX viability at different concentrations (0.1 – 1000 μg/mL). Results are expressed as mean ± standard deviations ($n = 3$). Different letters ([a], [b]) indicate significant differences between different concentrations of the same cell line ($p < 0.05$).

Several studies conducted with goji berries extract focused on purifying the compound that constitutes the highest proportion of the fruit. Wang et al. (2021) tested the influence of *L. barbarum* polysaccharides (LBP) in Caco-2 cells across a concentration range between 10 and 100 μg/mL (Y. Wang et al., 2021). In all cases, the viability results were above 90 %, suggesting the absence of toxicity. In another study (Mao, Xiao, Jiang, Zhao, Huang, & Guo, 2011), the effect of LBP in a wider range of concentrations (0 to 1000 μg/mL) and with 5 days of exposure was screened. The authors reported that after 5 days, LBP inhibited the cell viability in concentrations between 200 and 1000 μg/mL, confirming a cell viability decrease of 50 % for the concentration of 900 μg/mL (2 days of exposure) or 600 μg/mL (4 days of exposure). To the best of our knowledge, this is the first study that explores the effects of the whole extract in Caco-2 and HT29-MTX cell lines.

3.4.1. *Intestinal permeability model*

The co-culture model of Caco-2 and HT29-MTX closely mimics the *in vivo* intestinal environment, being an *in vitro* simple, less expensive, without ethical critical, and highly reproducible approach to evaluate the absorption of bioactive compounds (Sarmento et al., 2012). Caco-2 cells mimic the human colon due to the presence of microvilli and tight junctions, as well as numerous transporters, enzymes, and nuclear receptors, whereas HT29-MTX can simulate goblet cells, allowing the muco-adhesion of carrier systems to be evaluated (Silva, Almeida, et al., 2022).

In this work, a co-culture intestinal model was performed to assess the permeability of the compounds present in the optimal goji berries extract. Based on the MTT assay results, the co-culture model was exposed to the optimal extract in a concentration of 500 μg/mL. The various phytochemical compounds detected and quantified at the different time points are described in Fig. 4, as well as in Supplementary Table 1.

Supplementary Fig. 2 represents the acquired chromatograms after 240 min. To the best of our knowledge, this is the first investigation that screened the permeation of the different goji berries extract bioactive compounds on an intestinal model.

Glu-lycibarbarspermidine F (isomer 2) was the compound detected in higher amounts in most of the timepoints, reaching an intestinal permeability of 73.70 % at the final timepoint (240 min), followed by 3,5-dicaffeoylquinic acid (52.66 %) and isorhamnetin-3-*O*-rutinoside (49.31 %). Nevertheless, 3,5-dicaffeoylquinic acid achieved the highest permeation (16.56 %) in the first timepoint (15 min).

Multiple parameters may influence the chemical intestinal permeation, such as concentration, molecular size, compound structure and hydrophilicity, permeation time, and TEER (González et al., 2019; Diana Pinto et al., 2023). For instance, 3,5-dicaffeoylquinic acid (515 m/z), isorhamnetin-3-*O*-rutinoside (623 m/z), and rutin (609 m/z), are relatively small, freely moving through the intestinal barrier, achieving higher permeations at the final timepoint (52.66, 49.31, and 34.72 %, respectively). Nonetheless, the molecular size of the bioactive compounds present in the optimal extract depends on their glycosylation. The addition of sugar molecules increases the molecular weight and alter the compound three-dimensional structure and overall size (Qian et al., 2021). These modifications might prevent molecules from passing the cellular layer by common molecular transport (Sarmento et al., 2012), justifying the absence of kaempferol-3-*O*-rutinoside in the

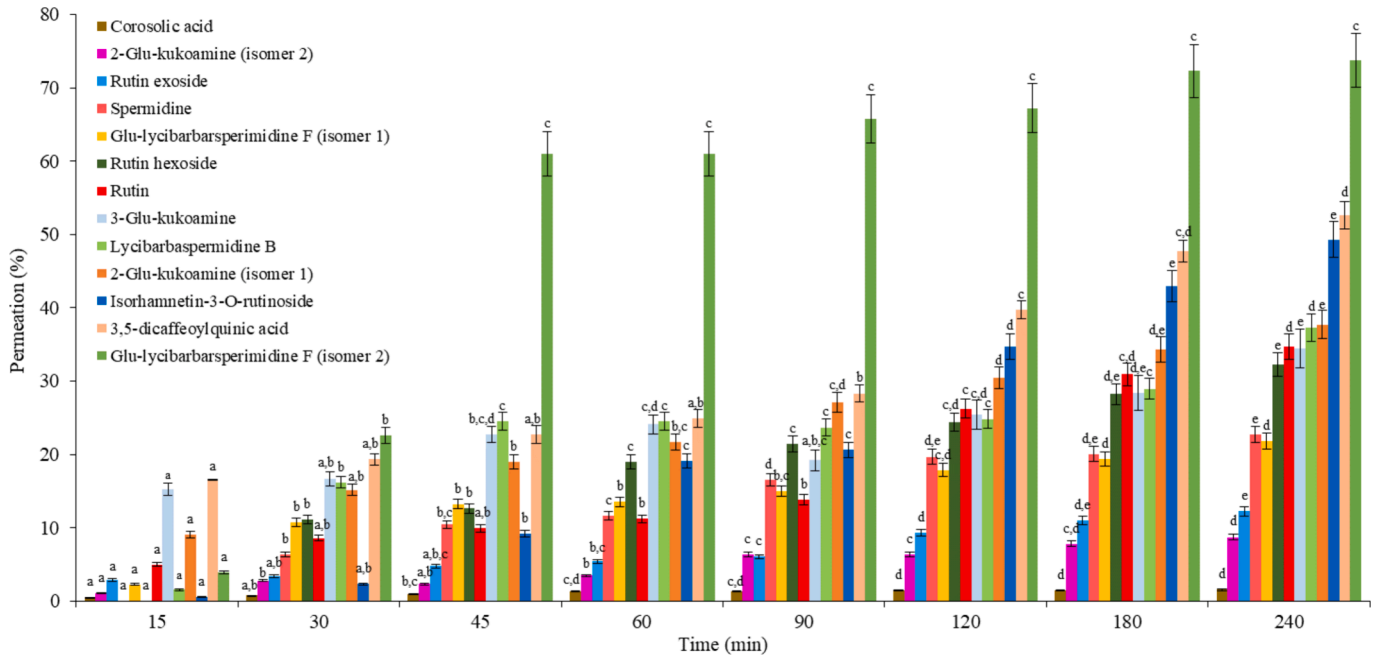

Fig. 4. Permeation percentages of the compounds from the optimal *L. barbarum* berries extract detected in the intestinal permeation assay at different timepoints. Results are expressed as mean ± standard deviations ($n = 3$). Different letters ($^{a, b, c, d, e}$) indicate significant differences between different timepoints of each compound ($p < 0.05$).

permeates.

On the other hand, glycosylation makes molecules more hydrophilic (Qian et al., 2021). This characteristic, along with the incorporation of HT29-MTX cell line in the co-culture model, is responsible for the improvement of the paracellular permeability of some compounds (González et al., 2019), such as glu-lycibarbarspermidine F (isomer 2), isorhamnetin-3-*O*-rutinoside, and 2-glu-kukoamine (isomer 1) that reached permeation percentages of 73.79, 49.31 and 37.73 %, respectively, at the final timepoint. Oppositely, hydrophobic compounds such as corosolic acid (Qian et al., 2021), displayed lower permeations at the different timepoints (ranging from 0.54 – 1.55 % from 15 to 240 min), despite the relatively high concentration that was applied in the apical side of the co-culture model (83.23 mg/100 g dw).

It should also be noticed the distinctive qualities of the several compounds present in the optimal extract along the intestinal permeability assay. Spermidine is an example since it has the ability to efficiently exploit specialized polyamine transporters found in intestinal cells to aid its absorption (Uemura, Stringer, Blohm-Mangone, & Gerner, 2010). This may not only justify the permeation percentage achieved at the end of the assay (22.75 %), but also support the high permeation (73.70 %) of glu-lycibarbarspermidine F (isomer 2).

There are also some phytochemical compounds that may act antagonistically and obstruct the absorption of other ones. For example, kaempferol and kaempferol glycosides, such as kaempferol-3-*O*-rutinoside, have been associated with antioxidant and anti-inflammatory capacities involved in the maintenance of enterocytes tight junctions, specifically by strengthening the tight junctions barrier in Caco-2 cells (Suzuki, Tanabe, & Hara, 2011).

The possible entrapment inside intestinal cells may also justify the low permeation observed for some compounds (González et al., 2019; Silva, Almeida, et al., 2022). For example, cells under oxidative stress have a greater tendency to take up bioactive compounds that possess antioxidant properties (González et al., 2019). Previous studies have demonstrated that rutin, chlorogenic acid, caffeic acid, and their derivatives, are effective antioxidants in human colon cell lines such as Caco-2 and HT29-MTX (Jiménez-Moreno et al., 2019; Wu et al., 2016). Furthermore, since the intracellular conditions are different from the extracellular ones, some compounds may be metabolized inside cells, not being detected in the permeation samples (Silva, Almeida, et al., 2022). This may justify the limited permeation observed for rutin exoside (12.24 %), 2-glu-kukoamine (isomer 2) (8.74 %), and corosolic acid (1.55 %), despite their relatively high presence in the optimal extract (12.85, 10.22, and 83.23 mg/100 g dw, respectively).

In this study, the TEER was measured for 21 days (Fig. 5 (I)) to guarantee the integrity and permeability of the model (Silva, Almeida, et al., 2022), as well as during the permeability assay (Fig. 5 (II)) to ensure the viability of the process. The values increased until the 10th day (198 ± 2 Ω/cm^2), supporting cell growth, and remained stable until the 21st day (177 ± 10 Ω/cm^2). On the 21st day of the experiment, during the permeability assay, the TEER values ranged between 176 Ω/cm^2 and 197 Ω/cm^2.

Silva et al. (2022) and Pinto et al. (2023) obtained comparable results when evaluating the permeation of *Actinidia arguta* leaf extract and *Castanea sativa* Mill. shell extract (Silva, Almeida, et al., 2022) (Diana Pinto et al., 2023), respectively, using a similar co-culture methodology. Nevertheless, as expected, these values are lower than other studies that only used Caco-2, since the mucus-secreting HT29-MTX cells modulate the Caco-2 tight junctions and, consequently, allow larger intercellular spaces between Caco-2 and HT29-MTX (Diana Pinto et al., 2023).

4. Conclusions

In the present study, the extraction of bioactive compounds from goji berries using the UAE technique was successfully achieved. The optimal extraction conditions that led to a maximization of the TPC and antioxidant/antiradical activities were optimized by RMS, with the optimal extract demonstrating an excellent composition in phytochemical compounds (glu-lycibarbarspermidine F (isomer 2) (126.72 mg/100 g dw), 2-glu-kukoamine (isomer 2) (104.96 mg/100 g dw), and rutin (19.27 mg/100 g dw)). These compounds were further associated with antioxidant and antiradical activities, particularly regarding ROS and RNS. Moreover, the optimal extract was classified as safe in intestinal cell lines in a concentration below 500 μg/mL. Most important, the intestinal permeability assay attested the significant permeation of the

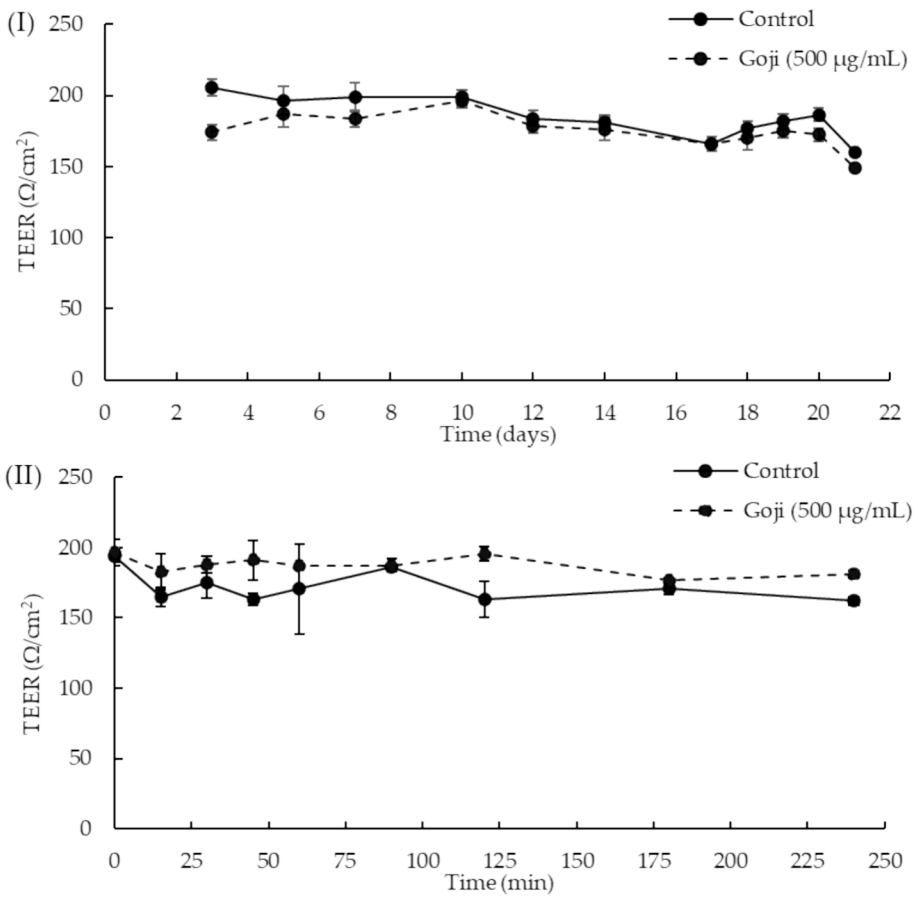

Fig. 5. TEER measurements during 21 days of co-culture cell model in Transwell™ membranes (I) and during the 240 min of the permeability assay (II). Results are expressed as mean ± standard deviations ($n = 3$) of two independent experiments.

principal compounds present in the optimal *L. barbarum* berries extract, with glu-lycibarbarspermidine F (isomer 2) (73.70 %), 3,5-dicaffeoylquinic acid (52.66 %), isorhamnetin-3-O-rutinoside (49.31 %), 2-glukukoamine (isomer 1) (37.73 %), and lycibarbarspermidine B (37.27 %) being the main ones. Based on the overall results, the health advantage of the employment of *L. barbarum* berries extracts in nutraceutical industry was successfully proved. Further studies should be focused on assess the antimicrobial and anti-inflammatory properties of the optimal extract, its stability over time, the exploration of encapsulation tolls to improve the intestinal absorption, and the employment of advanced *in vitro* cellular models to validate the biological activities already reported.

CRediT authorship contribution statement

Filipa Teixeira: Writing – original draft, Software, Methodology, Investigation, Formal analysis. **Ana Margarida Silva:** Methodology, Investigation, Formal analysis. **Stefania Sut:** Methodology, Investigation, Formal analysis. **Stefano Dall'Acqua:** Methodology, Investigation, Formal analysis. **Oscar L. Ramos:** Methodology, Investigation, Formal analysis. **Alessandra B. Ribeiro:** Methodology, Investigation, Formal analysis. **Ricardo Ferraz:** Supervision, Methodology, Investigation, Formal analysis. **Cristina Delerue-Matos:** Resources, Methodology. **Francisca Rodrigues:** Writing – review & editing, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Conceptualization.

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.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodres.2024.114502.

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