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Blackcurrant press cake by-product: Increased chemical bioaccessibility and reduced antioxidant protection after *in vitro* simulation of gastrointestinal digestion

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ABSTRACT

This study describes the bioaccessibility in terms of total phenolic content (TPC) and antioxidant capacity before and after *in vitro* digestion from blackcurrant press cake extracts (BPC) and the bioactivity in cell culture, human erythrocytes as well as the *in silico* analysis. Chemical analysis of BPC presented an increase in TPC (270%) and anthocyanins (136%) after *in vitro* digestion, resulting in an improvement of antioxidant activity (DPPH 112%; FRAP: 153%). This behavior may be related to the highest activity of cyanidin-3-rutinoside, as confirmed by *in silico* analysis. The digested BPC did not exert cytotoxicity in cells and showed less antioxidant activity against the oxidative damage induced in endothelial cells and human erythrocytes compared to the non-digested extract. The results raise a question about the reliability we should place on results obtained only from crude samples, especially those that will be used to produce foods or nutraceuticals.

1. Introduction

Blackcurrant (*Ribes nigrum* L.) is a dark-colored berry native to Northern–Central Europe and Asia and belongs to the family Grossulariaceae. This fruit is commonly used to produce juice, juice concentrate, and extract is widely utilized as food ingredients because of its potential effect on human nutrition and health (Diaconeasa et al., 2015; Olejnik et al., 2018; Xue et al., 2022). It has also been used as a nutraceutical to regulate or even alleviate various diseases once blackcurrant is a valuable source of carbohydrates and biologically active components, primarily polyphenols (mostly anthocyanins, flavonols, and phenolic acids), carotenoids, pectin and other fibers, ascorbic acid, and minerals (Pap et al., 2021). Among its emerging health benefits, are antimicrobial, antiproliferative (Xue et al., 2022), anti-inflammatory, and hypoglycemic activities (Xu et al., 2018; Granato et al., 2022), cardioprotective effects (Gubina-vakulyck et al., 2018) and suppression of colorectal tumorigenesis (Olejnik et al., 2018), the antioxidant activity is highlighted in all these conditions (Xu et al., 2018; Xue et al., 2022).

Overall, blackcurrant-based products have been turned into a worldwide trend due to their nutritional and bioactive benefits, the prominent European blackcurrant production of 153 028 metric tons/year, besides the seek for lesser-known fruits with authentic flavors (Granato et al., 2022). These factors lead to increased marketing and innovation efforts, which although enabling the larger scale for commercial purposes, also generate by-products in the same proportion, such as the blackcurrant press cake (BPC). BPC is obtained from the fruit pressing procedures and represents the impressive amount of 24 ± 4 % of berry mass (skins, seeds, and meat) with 38 % dry matter content. In terms of composition, the BPC presents ten different types of

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carbohydrates (which fructose, glucose, sucrose were the most abundant ones), representing 482 mg/g dw (Pap et al., 2021), 4.77 g/100 g of crude protein (richer in protein as compared to the 3 % of the whole fruit), 4.25 g/100 g of crude fat (monounsaturated and polyunsaturated fatty acids), and 15.5 g/100 g of fiber, compared to other berries (Blejan et al., 2023). It also presents a high content of anthocyanins, flavonols, and hydroxycinnamic acids (Granato et al., 2022; Blejan et al., 2023). Taking all these findings together, BPC is a valued product. It fills the proposals of the bio-circular economy and the Sustainable Goals of the United Nations – Goal 12 – Sustainable Consumption and Production (UN – 2015). Considering the large volume of discarded material generated by the food production sectors, the reuse of agri-food by-products, such as BPC, represents a considerable challenge for the future perspective of a green and sustainable economy (Pap et al., 2021).

To address this issue, we attempt to bridge the chemical and biological effect profile to subsidize using a food ingredient's initial residue from food processing. When dealing with bioactive compounds, especially anthocyanin sources, digestion transformations and bioaccessibility should be considered once they are highly affected by their chemical structure, the food matrix, and digestive system conditions. The phenolic compounds' bioaccessibility and breakdown rates during gastrointestinal digestion should be considered when estimating their phenolic content and antioxidant activity (David et al., 2019). However, as far as we know, the blackcurrant antioxidant bioactivity has been demonstrated primarily for pure compounds or crude extracts or juices (Diaconeasa et al., 2015; Gubina-vakulyck et al., 2018; Duan et al., 2020), and there are no cell-based studies available considering the modifications that occur throughout the digestive tract and, ultimately, after *in vitro* digestion. Although multiple research projects have focused on the benefits of phenolic structures *in vitro,* their bioactivity and clinical relevance are hypothetical and, sometimes, questionable, once several compounds and their metabolites tend to break down or be transformed in the intestine and may not be achieved in the human body, leading to the loss of this bioactivity (Granato, 2023b; Herrera-Balandrano et al., 2023). Notably, not all compounds are affected in the same way, and the differences in their bioactivity largely depend on their interactions in the food matrix, their shape, and structure, which influences their stability or interconversions (Burgos-Edward *et al.*, 2020; Herrera-Balandrano et al., 2023). In this sense, we focused on ascertaining the phenolic profile, the biological potential, and the safety of this BPC, as well as its digestive forms, considering the chemical transformations from the digestion, providing a unique view of the blackcurrant by-product as a valuable source of antioxidant phenolics for the pharma-food industry.

2. Materials and methods

2.1. Chemicals and cell lines

Gallic, chlorogenic, and ascorbic acids, Folin-Ciocalteu's phenol reagent, dichloro-dihydro-fluorescein diacetate (DCFH-DA), 3–4,5 dimethylthiazol-2, 5 diphenyl tetrazolium bromide (MTT), Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12 Ham (DMEM), and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (São Paulo, Brazil). 2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical and 2,4,6-Tris(2-pyridyl)-s-triazine (TPTZ) were obtained from Sigma-Aldrich-Merck (Darmstadt, Germany). Standards of cyanidin-3-*O*-glucoside, cyanidin-3-*O*-rutinoside, delphinidin-3-*O*-glucoside, and delphinidin-3-*O*-rutinoside were obtained from Extrasynthese (Genay, France). The other reagents were of analytical grade. Aqueous solutions were prepared using ultrapure water (Millipore, São Paulo, Brazil). Caco-2 (colorectal adenocarcinoma epithelial cells), HepG2 (Human hepatoma carcinoma cells), and EA.hy926 (Human vascular endothelial cell line) cell lines were purchased from Rio de Janeiro Cell Bank (Rio de Janeiro, Brazil).

2.2. Obtaining the raw material and extraction

Blackcurrant press cake (moisture content 49.6 %) was obtained from a berry processing company in Suomussalmi, East Finland. Fresh pomace was dried in an industrial vacuum dryer at a temperature below 60 °C until a moisture content < 10 % was obtained. The rough composition of the pomace is 67 % dietary fiber, 3.5 % ash, 2.5 % lipids, 12 % protein, and 11 % other carbohydrates.

The aqueous extractions were performed using two independent replicates, based on the method described by Pap et al. (2021): 20 g of raw materials were extracted with 200 mL of distilled water under constant magnetic agitation and temperature monitoring for 60 min at 40 °C. Then, the extracts were centrifuged at 12.000*g* for 10 min and filtered using a qualitative filter paper.

2.3. Gastrointestinal digestion of BPC extract

The *in vitro* digestion was performed according to the COST INFOGEST protocol with modifications (Brodkorb et al., 2019), using amounts of extracts following the recommended serving size of small berries, including sequential oral, gastric, and intestinal digestion steps. Briefly, 5 g of BPC was subjected to oral phase (pH 7, amylase solution 14 U/mg), and incubated in a water bath (37 °C) at 110 oscillations/min for 2 min (Fig. 1). The gastric (pH 3, pepsin solution – 25 000 U/mL) and intestinal (pH 7, pancreatin – 800 U/mL and bile salts solution – 160 mM) phases were performed in sequence, also incubated in a water bath (37° C) at 110 oscillations/min for 2 h. The samples were centrifuged at 12.000 *xg* for 10 min to separate the soluble or bioaccessible and residual fractions. The supernatant was collected, and 50 % trichloroacetic acid (TCA) was used to inhibit the different enzymatic activities of the digested samples (2 % as a final concentration). Blanks were run in parallel and analyzed to discard interferences due to the reagents of the digestion process.

The bioaccessibility, which represents the percentage of compounds released from BPC, is determined by:

$$\text{Bioaccessibility (\%)} = \text{CA/CI x 100} \quad (1)$$

where CA is the concentration of the compound in the intestinal phase, and CI is the initial concentration of the compound in the crude BPC, expressed as a percentage.

2.4. Chemical composition, antioxidant activity, and electronic properties

2.4.1. Phenolic composition of BPC extracts

Total phenolic content was assessed using the Folin-Ciocalteu phenol reagent and gallic acid equivalent (GAE) as a standard ($R^2 = 0.998$), and the results were expressed as mg of GAE per 100 g of BPC (mg GAE/100 g). Anthocyanins and caffeoylquinic acids (chlorogenic acid and neochlorogenic acid) were determined in their native forms by high-performance liquid chromatography (Shimadzu LC-20T, Kyoto, Japan), using a diode detector array (DAD), and fluorescence detectors, degasser system, autosampler, and oven column, according to the method validated by Fidelis et al. (2018). The thiolytic degradation method followed by UHPLC-DAD-FLD was applied to determine condensed tannins (proanthocyanins).

2.4.2. In vitro antioxidant capacity

Different assays were used to characterize the antioxidant capacity of the BPC extract, assessed according to Fidelis et al. (2018): free-radical scavenging activity in relation to the DPPH radical and ferric-ion reducing antioxidant power (FRAP) were quantified, and the values were expressed as mg of ascorbic acid equivalent per 100 g (mg AAE/100 g, dw).

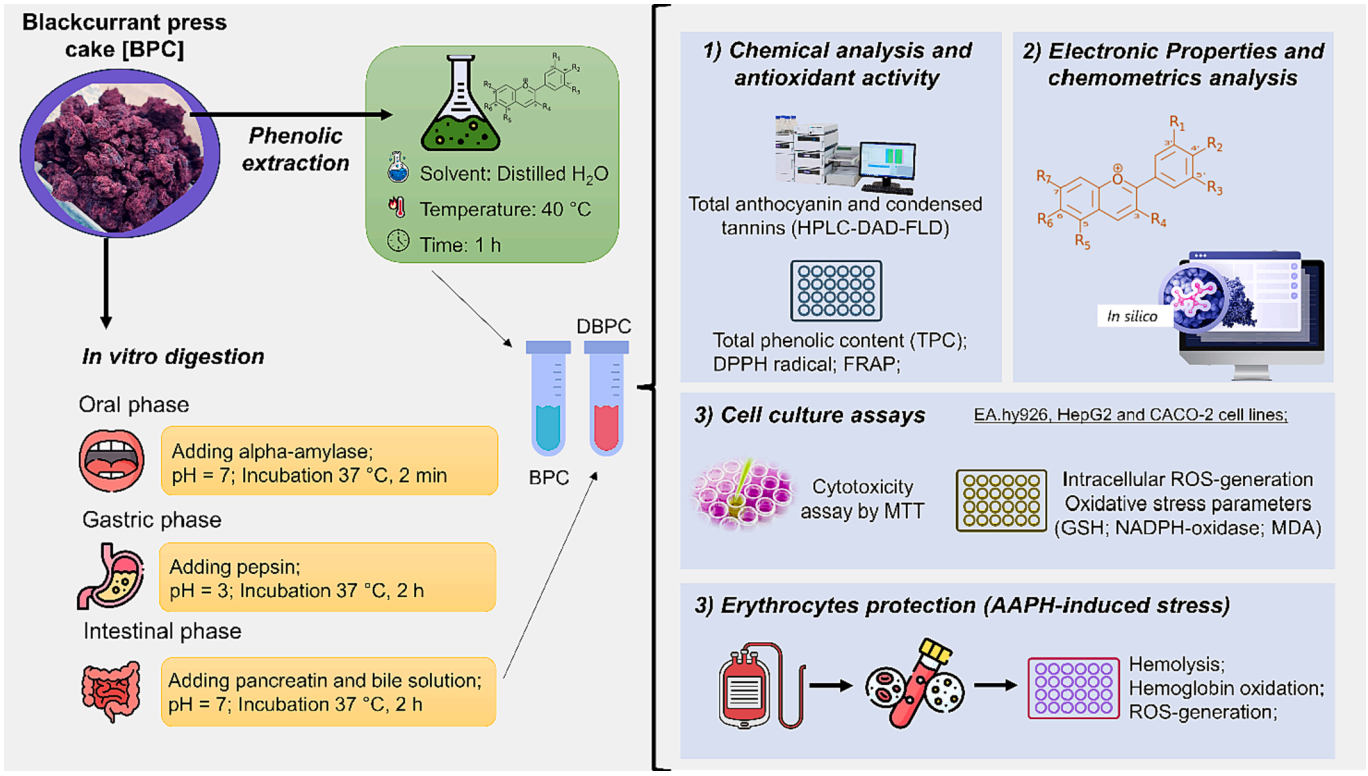


Fig. 1. Workflow for assessing the bioaccessibility of BPC extracts in chemical and *in silico* analysis, as well as the bioactivity in cytotoxicity, oxidative stress parameters in endothelial cell lines, and erythrocyte cellular antioxidant activity.

2.4.3. Electronic properties and chemometrics analysis of potential antioxidant activity

Chemometric analyses were performed according to the method of Maltarollo et al. (2019). The compounds used in the analysis had their 3D structures determined by conformational analysis utilizing the OMEGA 2.5.1.4 software (OpenEye Scientific Software, Santa Fe, NM). The electronic and molecular properties were calculated using the Spartan' 20 program (Spartan' 20, Wavefunction, Inc., Irvine, CA, USA) with the DFT method and B3LYP functional in combination with a 6–31 g* basis set. Then, the Hierarchical Clustering Analysis (HCA) and Principal Component Analysis (PCA) chemometrics were performed using the Euclidean distance as a similarity measure and the average binding method and using values of experimental antioxidant activities. To select the variables that best represent the antioxidant activity, a visual inspection of scatter plots was carried out, followed by hierarchical cluster analysis (HCA) and principal component analysis (PCA), which were performed using Chemoface 1.6 software. We selected the series Fernandes et al. (2013) reported as a control of anthocyanins and derivatives and further calculations of electronic and molecular properties. We employed a strategy similar to an earlier work (Maltarollo et al., 2019) by classifying the compounds according to various antioxidant activities using hierarchical clustering analysis and then comparing results from compounds reported in the literature with compounds identified in this work.

2.5. Cell culture and in vitro cytotoxicity evaluation

The *in vitro* cytotoxic effect of crude and digested BPC extracts was tested against EA.hy926, HepG2, and Caco-2 cell lines. The cell cultures were grown in Dulbecco's modified Eagle's medium (DMEM) at 37 °C under a humidified 5 % CO_2 atmosphere. The medium was supplemented with 10 % fetal bovine serum (FBS) and a mixture of penicillin (100 U/mL) and streptomycin (100 mg/mL) as antibiotics (1 %). The cells were seeded at 1 x 10^4 per well, and after 48 h of treatment with the crude and digested BPC extracts (5 – 100 μg GAE /mL), the cytotoxicity was determined using the MTT (0.5 mg /mL) colorimetric method for 4 h at 37 °C. The metabolically active cells reduced the MTT to blue formazan crystals, dissolved in 100 μL of DMSO, and the absorbance was measured at 570 nm. The IC_{50} (50 % cell viability inhibition), GI_{50} (50 % growth inhibition), and LC_{50} (50 % cell death) parameters were performed as described by Nascimento et al. (2023).

2.6. Reactive oxygen species (ROS) generation

Intracellular ROS generation was assessed using the DCFH-DA assay. Caco-2, HepG2, and EA.hy926 cells (6×10^4 per well) were treated for 1 h with crude and digested BPC extracts (2–50 μg GAE/mL) or 22,5 μmol /L of H_2O_2 (positive control) or respective blanks with culture medium with 2 % FBS (negative control). Following the treatment, H_2O_2 was added in the wells at 22.5 μmol/L diluted in HANKS solution, and the fluorescence intensity ($\lambda_{emission}$ = 538 nm and $\lambda_{excitation}$ = 485 nm) was measured (Nascimento et al., 2023).

2.7. Effect of BPC and DBPC on cellular antioxidant activity

EA.hy926 cell was seeded into 24-well plates at a density of 5×10^5 cells per well in 1000 μL of culture medium and incubated for 24 h at 37 °C. The cell line was treated with the extracts in different concentrations (5, 10, and 50 μg/mL) for 1 h, with or without hydrogen peroxide H_2O_2 (22.5 μmol/L). Following treatment, the medium was removed, and the cells were collected and resuspended in 250 μL of PBS. The activities of GSH, GSSG, total GSH, MDA, and NADPH oxidase were measured in triplicate, according to Pinheiro et al. (2016).

Briefly, lipid oxidation was determined by measuring thiobarbituric acid reactive substances (TBARS) using a fluorometric method, with excitation at 515 nm and emission at 553 nm, using 1,1,3,3-tetramethoxypropane as the standard. Lipid oxidation levels were expressed in malondialdehyde (nmol/mg). GSH levels were measured in duplicate

by colorimetric reaction with 5,5́-dithiobis 2-nitrobenzoic acid in 1 mol/L phosphate buffer (1 M, pH 7.4) and read at 412 nm. To measure the GSSG level, glutathione reductase and NADPH were added to the sample before measurement, reducing all GSSG to GSH, followed by measuring by the same colorimetric reaction. Then, the GSH concentration was a discount from the GSSG + GSH concentration. Glutathione was used as the standard (Lima et al., 2023).

Lucigenin chemiluminescence was performed to assess NADPH-dependent superoxide production. Briefly, lucigenin (5 μmol/L) was added to the cell homogenate. Following lucigenin addition, a concentration of βNADPH (12 μmol/L) was automatically added, and luminescence counts were continuously quantified at 37 °C using a microplate chemiluminescence reader (Synergy™ H1, Biotek, Agilent Technologies, Santa Clara, United States). Results were quantified by subtracting the background signal from the NADPH-driven signal, followed by normalization of the amount of cell protein. The results were expressed as lucigenin chemiluminescence/mg protein (Pinheiro et al., 2016).

2.8. Erythrocyte cellular antioxidant activity and protection assessment (HERYCA-P)

To evaluate the effect of BPC digestion on antioxidant activity and erythrocyte protection, blood (AB +) was obtained from a female volunteer (30-year-old, healthy, BMI < 25 kg/m^2) collected in test tubes with heparin (University of Limerick – Science and Engineering Research Ethics approval #2023_02_01_S&E), and written consent was obtained from the blood donor. Red blood cells (RBC, or erythrocytes) were isolated from the whole blood by successive washes with PBS (5 mmol/L, pH 7.35, NaCl 0.9 %) and centrifugations at 1200*g* for 5 min at room temperature until reached a clear supernatant. The supernatant was discarded, and the RBC were suspended with PBS till hematocrit 20 % and incubated with the samples (50, 100, and 150 μg GAE/mL), quercetin (25 and 50 μg/mL) or PBS (for the positive control). The erythrocytes' oxidation was induced with AAPH 200 mmol/L or PBS (negative control) for 120 min, 37 °C, and 150 rpm of orbital shaking. The hemolysis and intracellular ROS generation rates were evaluated according to a newly standardized protocol described by Cruz et al. (2024). The hemoglobin oxidation was investigated using the same procedure applied for the hemolysis assay, modifying only the wavelength to 540 and 630 nm. The hemoglobin oxidation rate was calculated by the following equation, where $A_{540\ nm}$ was the absorbance of the sample at 540 nm and $A_{630\ nm}$ was the sample's absorbance at 630 nm.

$$\text{Hemoglobin oxidation (\%)} = (A_{540\ nm}/A_{630\ nm})*100 \quad (2)$$

2.9. Statistical analyses

Analyses were performed in triplicate, and the results were expressed as means followed by the standard deviation. Differences between groups were compared using one-way analysis of variance (ANOVA), and means were compared using Tukey's test. When two samples were compared, an unpaired Student *t*-test was used. GraphPad Prism software version 8.0.c (GraphPad Software, Inc., San Diego, CA, EUA) was used for the statistical analysis of results and graphical representation. A probability value of $p < 0.05$ was considered significant.

3. Results and discussion

3.1. Effects of in vitro digestion on phenolic compounds and antioxidant activity of BPC

Herein, the chemical analysis of BPC extracts revealed that anthocyanins (197 ± 13 mg/100 g) were the most abundant components of all polyphenolics quantified in the undigested and digested BPC extracts (Table 1). Moreover, delphinidin-3-*O*-rutinoside was the predominant anthocyanin (on average 49.6 %) among the others, such as delphinidin-3-*O*-glucoside and cyanidin-3-*O*-rutinoside, delphinidin-3-*O*-rutinoside, and cyanidin-3-*O*-glucoside. Pap et al. (2021) found similar results analyzing BPC aqueous extract, which delphinidin-3-*O*-rutinoside represents 41 % of total anthocyanins present. Interestingly, these four major anthocyanins from blackcurrant by-product extract were also observed by Bishayee et al. (2010) in the whole blackcurrant fruits, which is a remarkable similarity once they represent 94–98 % of the total anthocyanin collectively and put in the spotlight this stream product as an essential ingredient. Over and above this higher similarity of the by-product with the fruit, and although anthocyanins are recognized by their low bioaccessibility Han et al. (2021), in our study, the total phenolic compounds and total anthocyanins in the BPC extract increased after *in vitro* simulated gastrointestinal digestion (DBPC) (from 685 ± 57 mg/100 g to 1850 ± 142 mg/100 g; and from 197 mg/100 g to 269 mg/100 g, respectively). Considering another berry and the same simulated gastrointestinal digestion protocol, Nascimento et al. (2023) found the total phenolic bioaccessibility from açai berry to be 25.17 %. Han et al. (2021) exemplify that blueberries may be a better source of bioaccessible anthocyanins when compared to raspberries and blackberries. Indeed, berries phenolic compounds possess individual bioaccessibility features, and herein, the bioaccessibility of caffeoylquinic acids reduced from 6.8 mg/100 g to 5.2 mg/100 g after *in vitro* digestion, although phenolic compounds were more bioaccessible. These variations are linked to the difference between their content in the food matrix, once phenolics can be covalently bound to indigestible components. In this sense, although little attention is paid to using by-products as a bioactive ingredient, there are promising results showing BPC as a source of highly bioaccessible phenolic compounds in special anthocyanins.

In contrast, the bioaccessibility of condensed tannin (CT) content, composed of a mixture of procyanidins and prodelphinidins, decreased to 15 % even though the composition proportion was kept unaltered after *in vitro* digestion. Tannins are partially metabolized and available for absorption at different sites in the gastrointestinal tract and the colon being the principal site of absorption. Due to their large size,

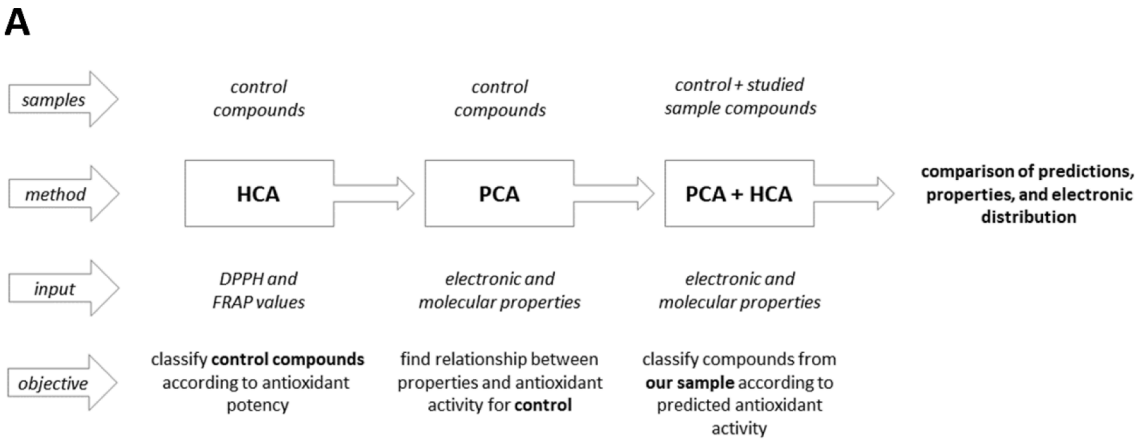
Table 1
Chemical composition of crude and digested blackcurrant press cake extracts.

Phenolic compounds	BPC (mg/100 g DM)	DBPC (mg/100 g DM) Bioaccessibility (%)[#]
TPC (mg GAE/100 g)	685 ± 57	1850 ± 142* (270 %)
Total Anthocyanins (mg/100 g)	197 ± 13	269 ± 15.7* (136 %)
Delphinidin-3-Glucoside	26 ± 2	51.5 ± 3.5* (198 %)
Delphinidin-3-Rutinoside	101 ± 6.5	129 ± 6.6* (128 %)
Cyanidin-3-Glucoside	8.4 ± 0.3	9.4 ± 0.3* (112 %)
Cyanidin-3-Rutinoside	60.6 ± 4.8	79.2 ± 5.9* (131 %)
Condensed tannins (mg CE/100 g)	142.5 ± 5.9	20.9 ± 2* (15 %)
Average Degree of Polymerization	11 ± 0.54	7.2 ± 0.5* (65 %)
Procyanidin / Prodelphinidin	37 / 63	35 / 65[n.s.] (95 %/103 %)
Caffeoylquinic Acids	6.8 ± 0.45	5.2 ± 0.2* (76 %)
Neochlorogenic acid	1.2 ± 0.02	0.8 ± 0.04* (67 %)
Chlorogenic acid	5.6 ± 0.42	4.4 ± 0.16* (79 %)
Antioxidant activity		
DPPH (mg AAE/g)	402 ± 16	854 ± 14*
FRAP (mg AAE/g)	675.8 ± 16	1708 ± 17*

Note: BPC = Blackcurrant press cake, DBPC = digested blackcurrant press cake, TPC = total phenolic content, DPPH = 2,2-diphenyl-1-picrylhydrazyl, FRAP = Ferric Reducing Antioxidant Power, GAE = gallic acid equivalent, CE = catechin equivalent, AAE = ascorbic acid equivalent. Values are expressed as means followed by the standard deviation (n = 4) [#]Bioaccessibility (%) = BPC/DBPC × 100. The difference was evaluated by an unpaired Student-*t*-test and indicates a statistical difference, n.s. indicates no significance ($p \leq 0.05$).

proanthocyanidins or condensed tannins are generally less bioaccessible than smaller phenolics. The bioaccessibility and biological effects of ingesting condensed tannins depend on their degree of polymerization and solubility. Therefore, depolymerization is essential, resulting in smaller monomeric and dimeric species with potentially higher bioaccessibility (Adarkwah-Yiadom & Duodu, 2017). A high degree of BPC tannins polymerization has already been reported (25.6 ± 1.3) in crude extract (Pap et al., 2021). In comparison, the average degree of polymerization was reduced by 35 % in our digested sample (from 11 ± 0.54 to 7.2 ± 0.5). Thus, herein, we hypothesized that reducing the degree of polymerization in DBPC indicates that condensed tannins in BPC passed through depolymerization during the *in vitro* digestion process.

Some data in the literature suggests that gastrointestinal *in vitro* digestion is considered a process that allows mechanical, biochemical, and enzymatic interactions. Enzymatic extraction of macronutrients, micronutrients, and phytochemicals (Burgos-Edwards et al., 2020) highly affects the extraction and bioaccessibility of compounds (Olejnik et al., 2018), including a considerable structural modification, which is responsible for affecting its biological effect (Brown et al., 2012). Considering that the entire digestive process affected the bioaccessibility



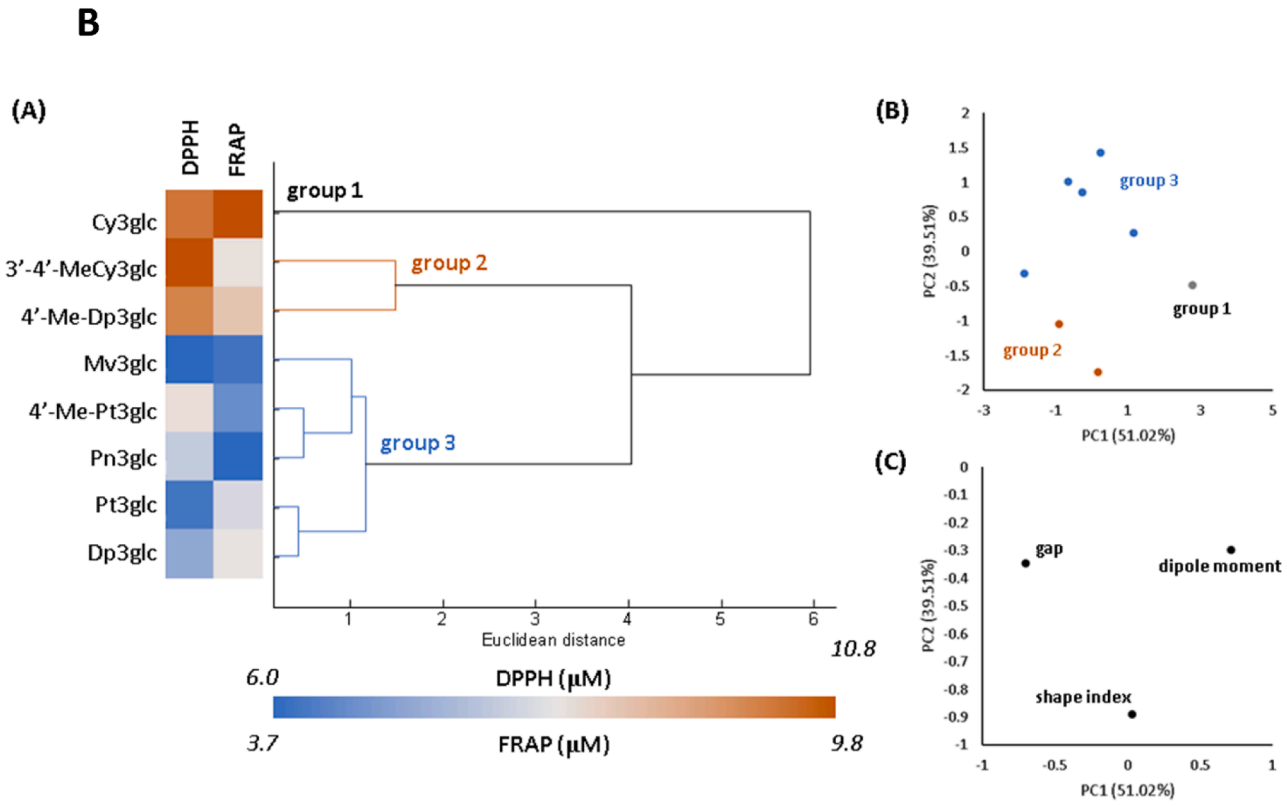


Fig. 2. Chemometric analysis. **A.** Fluxogram describing the methods, data, and objectives required in each step of the developed pipeline. **B.** HCA of control samples with antioxidant activities (A). Loadings plot from PCA with control samples (B) and score plots of variables used in this step (C) indicating the importance of each selected descriptor. Cy3glc: cyanidin-3-glucoside; 3'-4'-MeCy3glc: 3'-4'-methyl-cyanidin-3-glucoside; 4'-Me-Dp3glc: 4'-methyl-delphinidin-3-glucoside; Mv3glc: malvidin-3-glucoside; 4'-Me-Pt3glc: 4'-methyl-petunidin-3-glucoside; Pn3glc: peonidin-3-glucoside; Pt3glc: petunidin-3-glucoside; Dp3glc: delphinidin-3-glucoside.

of BPC compounds, the percentage of phenolic compounds and anthocyanins increased by around 270 % and 136 %, respectively. The higher TPC values may also be attributed to the hydrolysis of flavonoid glycosides to release aglycones during the digestion process and to the presence of nonphenolic reducing substances such as peptides from the hydrolysis of proteins and sugars from the hydrolysis of starch, once active enzyme digestion seems to improve accessibility of phenolic compounds (Chiang et al., 2013; Adarkwah-Yiadom & Duodu, 2017). The bioaccessibility of condensed tannins and caffeoylquinic acids decreased to 15 % and 76 %, respectively. The degradation of phenolic compounds during *in vitro* gastrointestinal digestion is mainly ascribed to the intestinal environment's pH, digestive enzymes, the ability to cross membranes, biliary acids, and food matrix, which can modify the chemical structures, resulting in new compounds with different bioavailability and biological activity (Herrera-Balandrano et al., 2023).

Scientific evidence highlights that phenolic compound content has been reported as the main contributor to the antioxidant effect (do Carmo et al., 2018). The improvement of the major components in the BPC extracts after *in vitro* digestion corresponded with higher antioxidant activity, proved according to FRAP (854 ± 14 mg AAE/100 g; 153 %) and DPPH (1708 ± 17 mg AAE/100 g; 112 %) analysis, as shown in Table 1. Similarly, Chiang et al. (2013) demonstrated that digestion increases the bioaccessibility of the antioxidants present in gooseberry fruit, affecting its antioxidant properties through the higher potency in scavenging DPPH radicals. Considering that BPC was processed through simulated intestinal digestion and its antioxidant capacity was higher after digestion, we suggest that a higher total phenolic content (e.g., including anthocyanins) in the digested sample also improved antioxidant activity.

3.2. Electronic properties and chemometrics analysis of potential antioxidant activity

The complete pipeline applied in this work is shown in Fig. 2A. From that first analysis, compounds were grouped into groups 1 and 2 (most antioxidant) and group 3 (less antioxidant) according to HCA [Fig. 2B (A)]. Then, we optimized the geometry of the 3D structure of compounds and calculated electronic structure using Spartan'20 software and molecular descriptors using DataWarrior, aiming to stabilize the most representative properties that correlate to antioxidant activities.

After selecting the properties with higher influence by visual inspection of scatter plots (two variables and samples colored by classes [Fig. 2B(B)], the following principal component analysis indicated that gap (energy of HOMO – the energy of LUMO), dipole moment and shape index are three properties able to distinguish compounds with potent antioxidant activities (groups 1 and 2) from most minor active compounds [Fig. 2B (B and C)). The gap is a property related to the electron transfer ability of a given molecule. Polarizability was described as and critical to distinguishing the antioxidant activity of flavonoids (Weber et al., 2006), and it is also related to HOMO/LUMO energies, as well as the gap (Arroio et al., 2010).

The dipole moment describes the overall electron delocalization of a molecule and, as all compounds are structurally similar, this property could be used to evaluate the influence of substituents on the electronic structure. In other words, dipole moment could be related to the orientation of intermolecular interactions of a specific compound (Das & Banik, 2020). Finally, the shape index codifies non-spheric compounds or perfectly spheric ones (ranging from 1 to 0, respectively). In that sense, the shape of compounds could affect how they interact with other molecules and, consecutively, interfere with their biological properties (Tripathi and Bankaitis, 2018). In this sense, the polarizability, shape, and electron transfer ability describe the interaction and interference of compounds in biological redox processes and, therefore, explain their role as cellular antioxidant agents.

After this analysis, we followed a complete comparison using HCA and PCA with selected properties using control compounds and the compounds identified in our samples (Fig. 3A-B). Two compounds from our samples are the same as those reported isolated by Fernandes et al. (2013): Cy3glc and Dp3glc. Therefore, we removed those compounds from the analysis in the following comparison. Interestingly, Cyan-3-rut was very similar to most active compounds, and all other three compounds present in our samples (Delph-3-rut, chlorogenic acid, and neochlorogenic acid) were considered outliers due to differences in selected descriptors. However, the dipole moment vector and HOMO and LUMO maps (Fig. 3C) indicated that the rings A, B, and C of anthocyanins are responsible for the electron transfer of antioxidant effect, and the comparison of the highest active and least active compounds presented similar electronic distribution. This is an expected feature since conjugated systems (e.g. aromatic rings) play a known role in the electronic effects of organic compounds. Chlorogenic and neochlorogenic acids were considered outliers; they are structurally distinct from anthocyanins, and potentially expected to be outside our model's applicability domain. Despite that, some studies (Yang et al., 2022; Corrigan et al., 2023) attribute antioxidant activity to those compounds and derivatives. In this sense, all compounds have antioxidant activity with changes in potency. Therefore, it may indicate a synergic effect of all anthocyanins and chlorogenic/neochlorogenic acids present in our samples.

3.3. In vitro cell-based cytotoxicity

Fig. 4A shows *in vitro* biological effects in a cell-based experiment of BPC extracts. First, the crude extract showed no cytotoxicity (LC_{50} > 100 μg/mL), and it did not interfere with the cell growth and viability (IC_{50} and GI_{50} > 100 μg/mL) in all cell lines tested (Caco-2, EA.hy926, and HepG2). In agreement with our results, Pap et al. (2021) found that BPC freeze-dried extracts also exhibited high IC_{50} and IG_{50} values (>2000 μg/mL) in all the cell lines tested (A549, HCT, and IMR90). Otherwise, Bishayee et al. (2010) treated human liver cancer cells (HepG2) with varying concentrations of blackcurrant extract, rich in delphinidin and cyanidin. They found a cytotoxicity effect at a concentration of 1.35 mg/mL. Similar results are demonstrated by Diaconeasa et al. (2015), who tested an anthocyanin sample purified from blackcurrant juice and found a dose-dependent reduction in viability of HeLa, A2780, and B16-F10 cell lines at concentrations between 200 and 300 μg/mL.

Regarding the DBPC extract, this exerted no cytotoxic activity related to HepG2 and Caco-2 cells (IC_{50}, GI_{50}, and LC_{50} > 100 μg/mL), indicating that very high concentrations are required to inhibit the proliferation of half of these cells, meaning low cytotoxicity of both crude and digested samples. Brown et al. (2012) also demonstrated no cytotoxic activity in colon cells when tested digested blackcurrant samples at concentrations between 0 and 50 μg/mL after 24 h. On the other hand, EA.hy926 has been most sensitive toward the DBPC extract since they presented estimated values of GI_{50} = 75 μg/mL, indicating that the cell proliferation was inhibited. This discrepancy hypothesized that the cumulative ROS induces a cellular redox imbalance and plays a central role in attenuating uncontrolled cell proliferation (do Carmo et al., 2018), as we demonstrate in the intracellular reactive oxygen species assay (item 3.5).

3.4. Intracellular reactive oxygen species (ROS) activity

In our study, as expected, the levels of induced ROS by hydrogen peroxide (H_2O_2) as positive control were higher than the spontaneous generation group (Fig. 4B). We highlight that crude BPC extract did not induce ROS production and, when exposed to high concentrations of H_2O_2, displayed a reduction of ROS, suggesting a protective and intracellular antioxidant effect against injuries from oxidative stress [Fig. 4B (A-C)]. Similar results are observed by Pap et al. (2021), who investigated BPC extract in colon cancer cells (HCT8) and found suppression of intracellular ROS in all concentrations tested (100–1000 μg/mL).

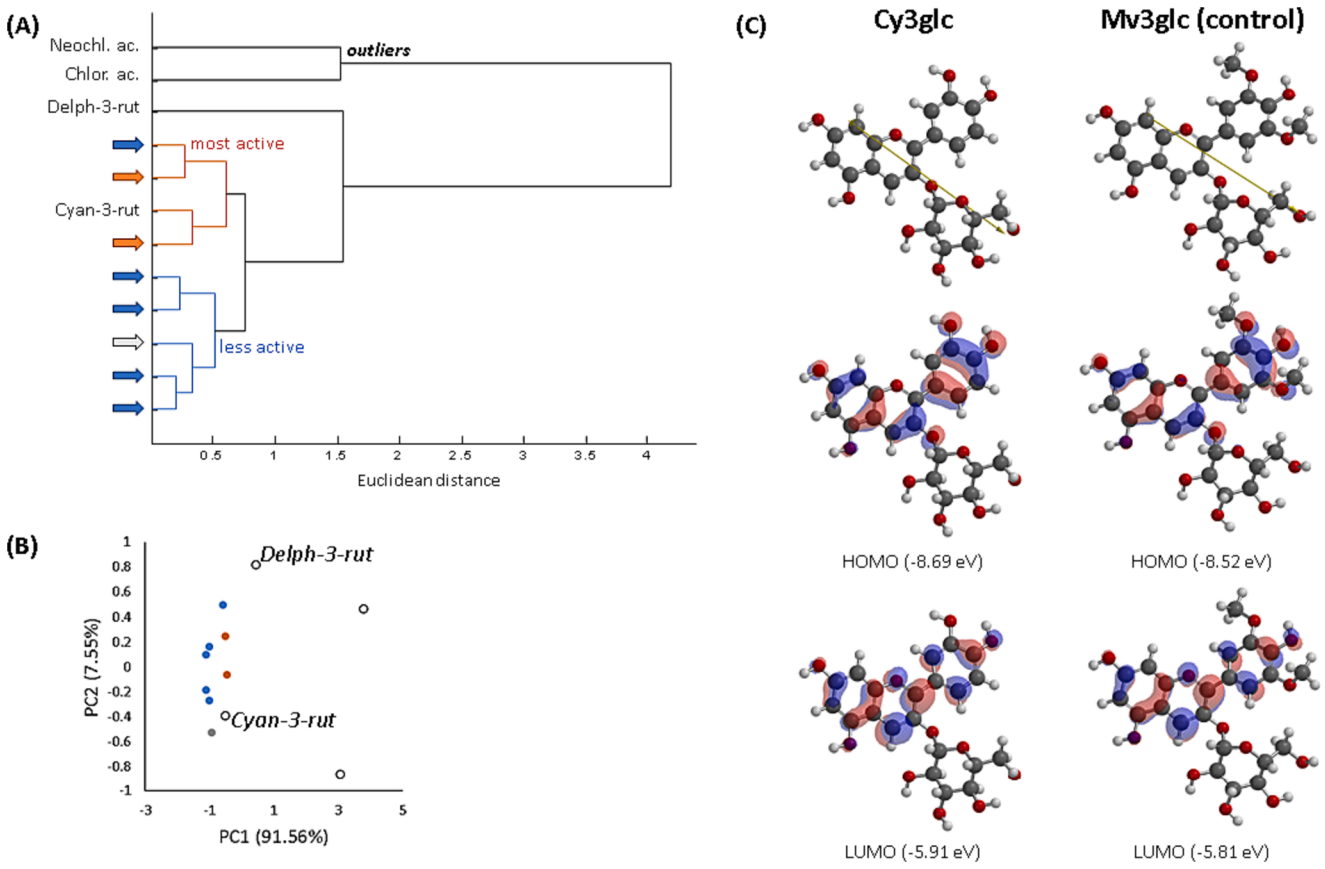


Fig. 3. HCA of control samples and compounds from studies samples with electronic properties (A). Loadings plot from PCA with control compounds and compounds from samples (B). Dipole moment vector (yellow arrow), HOMO, and LUMO plots (red and blue surfaces) of representative compounds (C). Cy3glc: cyanidin-3-glucoside; Mv3glc: malvidin-3-glucoside.

Interestingly, the digested extract was less effective in exerting antioxidant activity and protected against hydrogen peroxide induction in Caco-2 and HepG2 cell lines. A different behavior was found in the endothelial cell line (EA.hy926), in which the DBPC did not exert a protective effect once the levels of hydrogen peroxide were kept at the same level as the positive group (H_2O_2). Olejnik et al. (2018) showed that the gastrointestinal digested blackcurrant extract at the highest concentrations (20 mg/mL) also induced a substantial increase in the formation of ROS in colon cancer cells (HT-29).

Phenolic compounds' bioactivities are closely associated with their abilities to scavenge free radicals and stabilize reactive oxygen/nitrogen species (ROS/RNS). The number of hydroxyl groups, solubility, and the nature of substitutions on the aromatic rings play a crucial role in their bioactivity (Corrigan et al., 2023). Moreover, the low pH values contribute to anthocyanins' high stability, which is essential to their bioavailability and occurs in the chemical structure of a stable flavylium cation (David et al., 2019). Considering the final pH in the intestinal phase (pH = 7), we observed that the anthocyanins present in our samples after *in vitro* digestion presented low stability, and this is because of the anthocyanin conversion into a carbinol base at neutral pH (6–7). Furthermore, according to Herrera-Balandrano et al. (2023), anthocyanins with catechol (cyanidin) or pyrogallol (delphinidin) moieties on the B-ring have a remarkable single electron transfer property, enhancing their antioxidant effects. Considering that these anthocyanins were the most abundant in our digested sample (Table 1), our results clearly show that despite high bioaccessibility and *in vitro* antioxidant tests, these compounds can exert lower antioxidant activity in cell culture.

3.5. Cellular antioxidant activity

We assessed the lipid peroxidation levels by measuring thiobarbituric acid-reactive substances (expressed in terms of malondialdehyde; MDA), which is considered the primary molecular mechanism involved in membrane oxidative damage (Mas-Bargues et al., 2021). The hydrogen peroxide increases MDA levels in cells, as shown by the positive control group (H_2O_2), and treatment with both BPC and DBPC extracts did not induce lipid peroxidation in the endothelial cell line. They produced antioxidant effects against the induction of oxidative stress by H_2O_2 in all concentrations tested. However, the BPC exerted better antioxidant activity than DBPC (Fig. 5A), showing that the *in vitro* digestion reduced the potential antioxidant protector effect against lipid peroxidation.

As glutathione (GSH) plays a central protective role in catalyzing the reduction of organic hydroperoxides and lipid peroxides in membranes, we evaluated levels of GSH, GSSG, and total glutathione (Fig. 5B). Their antioxidant capacity can be attributed to the radical scavenging mechanism, which acts dynamically to reduce ROS/RNS and prevent more ROS generation (do Carmo et al., 2018). Herein, we demonstrated that the BPC exerts direct antioxidant activity, decreasing the oxidation of GSH induced by H_2O_2 addiction. In contrast, the DBPC did not exert a protective effect, maintaining the same consumption of GSH and the positive control. Considering that the bioaccessibility of phenolic compounds and anthocyanins was greater in the digested sample (1850 ± 142 mg/100 g and 269 ± 16 mg/100 g, respectively), we hypothesize that these bioaccessible phenolic compounds could present a dual-face behavior, acting as antioxidant or pro-oxidant agents, depending on the pH, presence of metals, and concentration of oxygen (do Carmo et al., 2018).

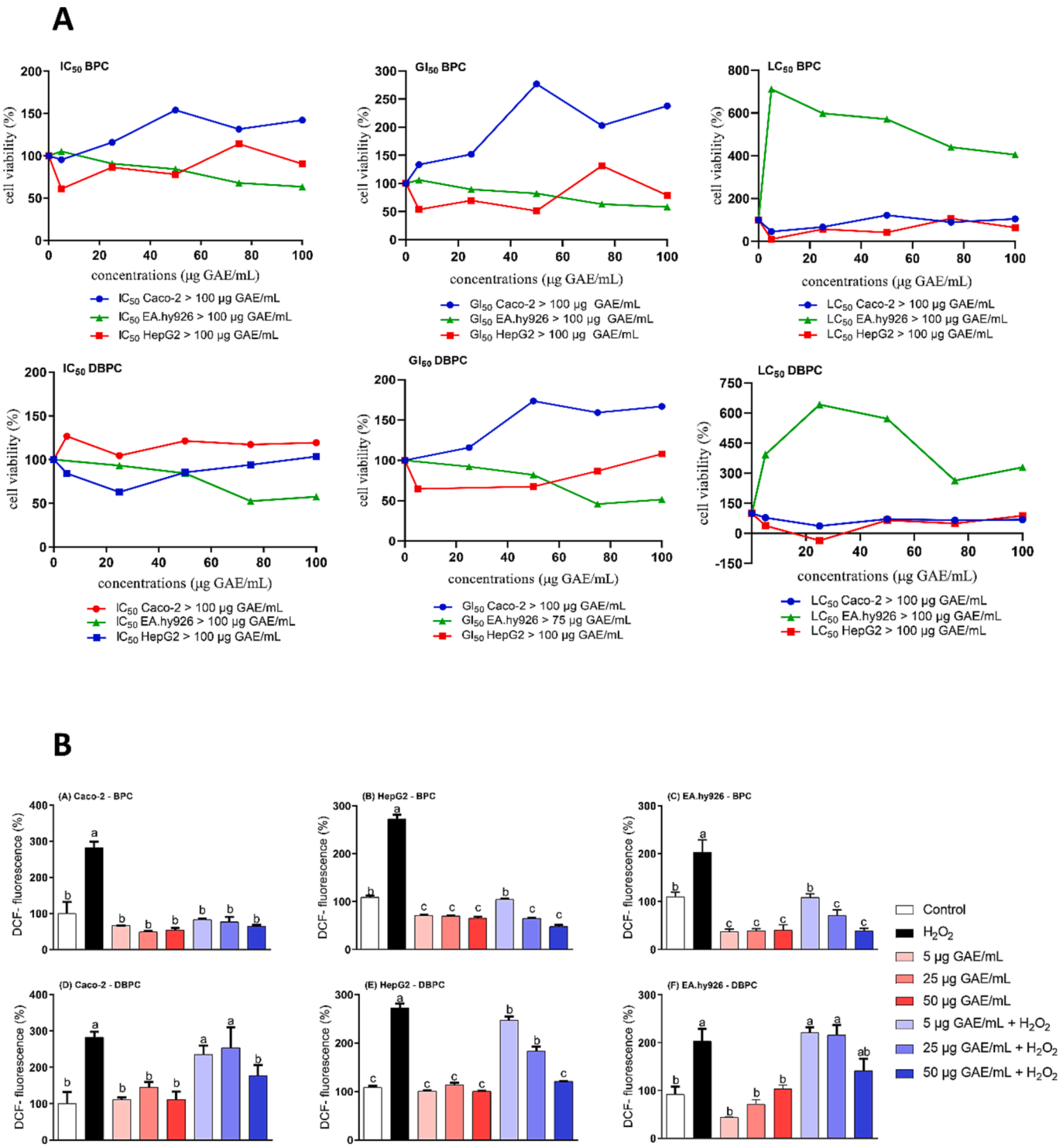


Fig. 4. Cell viability and Intracellular ROS-generation. **A.** Cell viability and representative evaluation of the concentration-dependent impact after 48 h exposure to the blackcurrant crude and digested extracts in Caco-2, HepG2, and EA.hy926 line cells. IC_{50} (concentration of the extracts that inhibit cell viability by 50 %); GI_{50} (concentration of the extracts that inhibit cell growth by 50 %); and LC_{50} (concentrations of the extracts that result in loss of 50 % cells). BPC: blackcurrant press cake; DBPC: digested blackcurrant press cake. **B.** Results of intracellular ROS measurement in Caco-2, HepG2, and EA.hy926 cells by spectrofluorimetric. Treatment with BPC and DBPC at 5–50 µg/mL. Quantitative data are the mean ± SD (n = 3). Different letters represent statistical differences ($p \leq 0.05$).

This maintenance of ROS levels could occur through changing activity to NADPH oxidase, a multi-subunit complex enzyme that is considered a key source of superoxide anion (O_2-), a ROS product in the vasculature via the AT1 receptor (Yousefian et al., 2019). As already noted in previous analyses, herein, the *in vitro* digestion process stimulated superoxide synthesis via NADPH oxidase or generated ROS by itself in a dose-dependent manner. At the same time, the BPC increased the enzyme in the highest concentration (50 µg GAE/mL) (Fig. 5C). All these data suggest that digestion impairs the antioxidant effect and inclines BPC to exert an oxidant effect as the tested concentration increases. In fact, according to Yousefian et al. (2019), the maintenance of ROS production has positive feedback, leading to a rise in NADPH

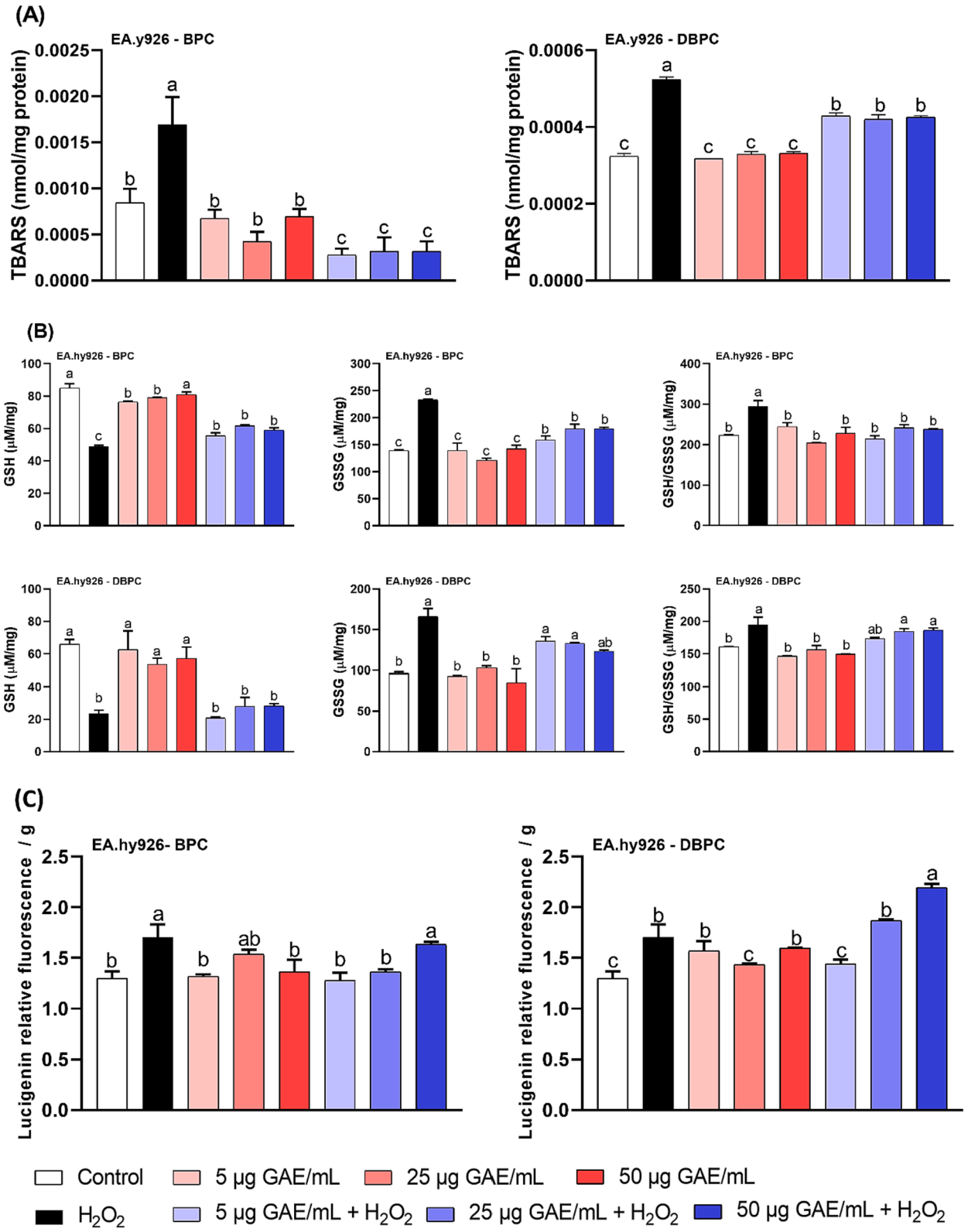


Fig. 5. Oxidative stress parameters. (A) MDA, malonaldehyde; (B) GSH, reduced glutathione; GSSG, oxidized glutathione; (C) NADPH oxidase, nicotinamide adenine dinucleotide phosphate (measured by lucigenin relative fluorescence). All values are mean ± SD (n = 3), and values with different letters are significantly different as determined by Tukey's test, $p < 0.05$. Note: BPC, blackcurrant press cake; DBPC, digested blackcurrant press cake; H_2O_2, hydrogen peroxide (22.5 µmol/L); GAE, gallic acid equivalent.

oxidase expression. A recent study of our research group also found that BPC, after *in vivo* digestion, increases the pro-oxidant effect of phenolic compounds when tested at high concentrations (15 %) in rats induced to colon cancer (Lima et al., 2023).

Although the *in vitro* digestion simulation has analytical limitations, as it is typically a static digestion model, it allows studying changes in food components during the oral, gastric, and intestinal phases (Han et al., 2021). It demonstrates the importance that we must consider, not only related to the nutritional content of the food but also the factors involved in the phenolics' bioaccessibility and bioactivity. Although in our study, the anthocyanins' bioaccessibility was greater after *in vitro* digestion, their bioavailability is limited by their sensitivity to a variety of digestive system conditions. According to Xue et al. (2022), anthocyanins have a low bioavailability, and around 85–90 % of anthocyanins reach the colon to be fermented by gut microbiota.

Steinert et al. (2008) evaluated the potential absorption of blackcurrant anthocyanins using the Caco-2 cell monolayer and no anthocyanins (delphinidin-3-*O*-glucoside, delphinidin-3-*O*-rutinoside, cyanidin-3-*O*-glucoside, and cyanidin-3-*O*-rutinoside) were identified in the basolateral solution, concluding that anthocyanin absorption and bioavailability may be influenced by translocation throughout the basolateral membrane. According to Xiao (2017), glycosylated flavonoids, such as anthocyanins, are highly water-soluble and poorly permeable in cell membranes, which hinders their diffusion into the cell. Moreover, the differences in the structural stability of flavonoids lead to changes in their content during *in vitro* digestion, resulting in differences in their activity (Xue et al., 2022). Overall, at first glance, it may seem misleading that despite the high bioaccessibility of phenolic compounds and the chemical antioxidant activity of DBPC being greater than that of BPC, this does not reflect an increase in cellular antioxidant activity after digestion. These observations are on par with the concept that *in vitro* antioxidant activity highly depends on the method and protocol used, cell line origin (e.g., human or murine), type and concentration of the oxidative stress inductor, and treatment time, among others (Granato, 2023a).

3.6. Protection of BPC extracts against AAPH-induced oxidation in RBC

We also evaluated the *in vitro* oxidative stress attenuation on human blood by BPC extracts by measuring three oxidative stress markers: hemolysis, hemoglobin oxidation, and intracellular ROS generation (Fig. 6). This oxidative stress was induced *in vitro* by AAPH, an oxidant decomposed thermally at 37 °C to generate alkoxyl and peroxyl radicals that lead to erythrocyte damage (Cruz et al., 2024). These radicals can oxidize membrane lipids and proteins (especially the band 3 protein), which promotes intracellular content leakage, and cytotoxicity known as hemolysis (Duan et al., 2020). Interestingly, both samples, BPC and DBPC, were effective at protecting the RBC against AAPH-induced hemolysis already at the lowest tested concentration (50 μg GAE/mL), presenting higher anti-hemolytic activity at 150 μg GAE/mL, reaching the level of negative control and quercetin 50 μg/mL (Fig. 6A).

This protection can be attributed to the samples' polysaccharides and phenolic compounds, especially chlorogenic acid and cyanidin-3-rutinoside, since these compounds protect RBC against oxidative hemolysis (Duan et al., 2020). In fact, according to the chemometrics analysis, cyanidin-3-rutinoside is the most active compound present in

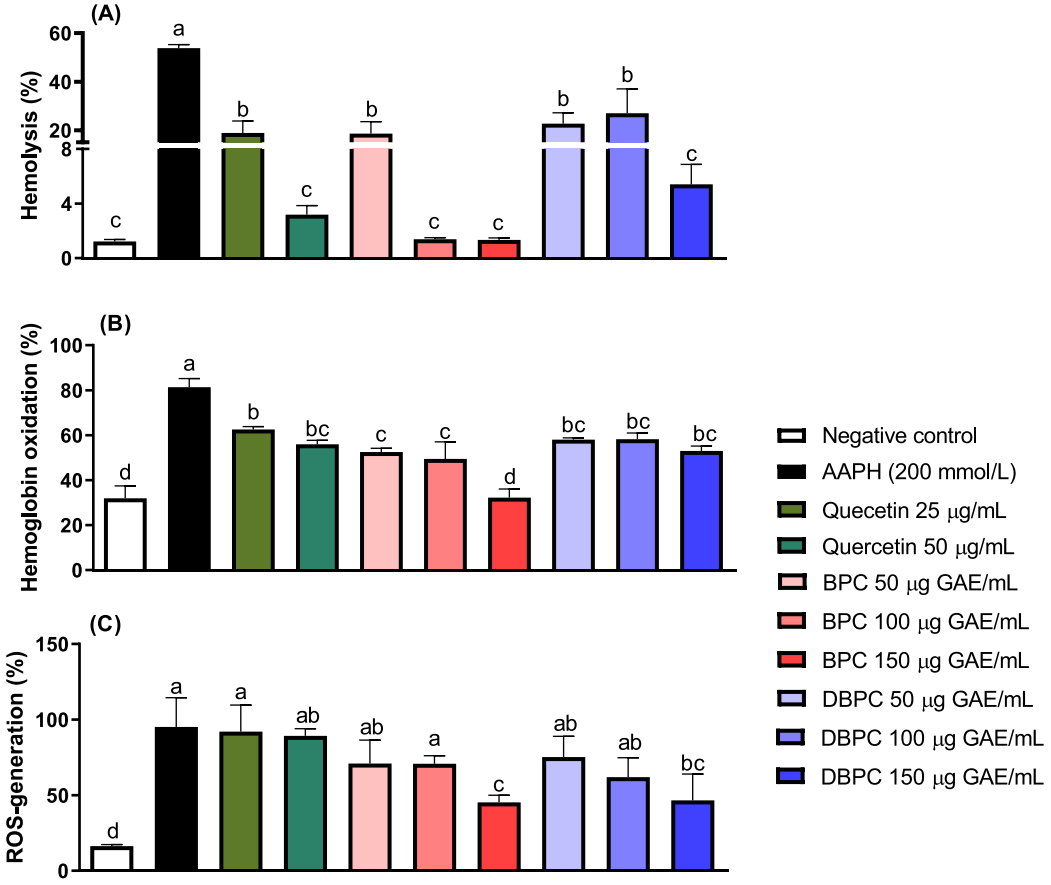


Fig. 6. (A) Effect of BPC extracts on blood hemolysis induced by2′-azobis(2-amidinopropane) dihydrochloride (AAPH); (B) Effect of BPC extracts on hemoglobin oxidation induced by AAPH; (C) Effect of BPC extracts on intracellular reactive oxygen species (ROS) generation induced by AAPH. Quantitative data are the mean ± standard deviation (n = 4). Different letters represent statistically significant differences ($p \leq 0.05$). Note: BPC, blackcurrant press cake; DBPC, digested blackcurrant press cake; GAE, gallic acid equivalent.

our samples (BPC = 60.6 ± 4.8 mg/100 g and DBPC = 79.2 ± 5.9 mg/100 g, respectively), which suggests an antioxidant activity and protection against AAPH-induced injury. Nonetheless, Han et al. (2021) demonstrate that the terminal metabolites of cyanidin-3-glucoside were identified in feces and urine and not absorbed into blood circulation. This result proves that, although anthocyanins have high biological activities, they are structurally unstable at the animal body's digestion, absorption, and metabolism before exerting their bioactivity.

Another consequence of the oxidative stress in RBC is the oxidation of Fe^{2+}-hemoglobin (or oxyhemoglobin) to Fe^{3+}-hemoglobin (or methemoglobin). Oxyhemoglobin presents two characteristic peaks in the 540 and 576 nm region; when it is oxidized to methemoglobin, a peak appears at 630 nm (Cruz et al., 2024). Herein, our samples were able to reduce the hemoglobin oxidation rates from 80 % (positive control) to 52.5 % (BPC) and 58 % (DBPC), an activity similar to the one shown by quercetin 25 and 50 μg/mL. Moreover, the BPC at 150 μg GAE/mL keeps this rate at the same level as the RBC basal (negative control) (Fig. 6B). The prevention of hemoglobin oxidation probably occurs by phenolic compounds scavenging reactive species and reducing the iron oxidized by these species (Cruz et al., 2024).

Regarding inhibiting AAPH-induced intracellular ROS generation in RBC (Fig. 6C), our samples were effective only at 150 μg GAE/mL, the highest tested concentration, indicating that the simulated digestion did not modify the sample's erythrocyte antioxidant activity. This effect is possibly associated with the phenolic compounds in the samples, especially anthocyanins, proanthocyanidins, chlorogenic acid, and neochlorogenic acid (Yang et al., 2022). Although this is the first time that there are results of the effects of BPC extracts on the inhibition of intracellular ROS in RBC, this blackcurrant extract's activity was already reported on lipopolysaccharide-stimulated RAW264.7 macrophages, where the blackcurrant extract (100 μg/mL) decreased the intracellular ROS generation to 30 % (Hui et al., 2021).

The fact that the DBPC did not modify the antioxidant activity agrees with what we described in the chemical analysis, where there is lower bioaccessibility of clonogenic and neochlonogenic acids in the digested sample. This behavior could be related to the fact that these acids were considered outliers in the chemometrics analysis and may have changes in the potency of the antioxidant effect. However, structurally, it is similar to anthocyanins and has synergistic effects. Thus, bearing these considerations, our results suggest that the different antioxidant behavior of the samples against the potentially harmful effects of AAPH and ROS oxidation reflects the particularity of each cell line related to the stability and bioavailability of the anthocyanins present in the digested sample.

4. Conclusion

The data from this study provides novel information about the *in vitro* digestion of BPC on antioxidant activity and the biological activities of their metabolites in an endothelial cell culture model after simulating physiological digestion conditions. The findings presented here indicate that following passage through the artificial gastrointestinal tract, BPC extract positively enhances the phenolic compounds, mainly anthocyanins. The chemometric analysis demonstrated that all the analyzed compounds present antioxidant activity, even with changes in potency, and cyanidin-3-rutinoside proved to be more active among the types of anthocyanins analyzed. In cell culture, only the endothelial cell line showed a reduction in cell proliferation in the digested BPC extract, mainly due to the high levels of oxidative stress generated by hydrogen peroxide induction and the NADPH oxidase activity. In addition, both tested samples effectively protected RBC against AAPH-induced oxidative stress, as BPC and DBPC could attenuate hemolysis, hemoglobin oxidation, and intracellular ROS generation. It is hypothesized that after digestion, the biological activity of BPC is dramatically changed due to considerable structural modification, which suggests that breakdown products and individual metabolites can alter cellular processes. Bearing this in mind, the greater consumption of BPC can be protective or harmful depending on the digestive stability of phenolic compounds, their bioaccessibility, bioavailability, and intestinal absorption. Considering that few articles use the digestion process in the production and the testing of samples, the results raise a question about the reliability we should place on results obtained only from crude samples, especially those that will be used to produce food, medicine, or nutraceuticals.

Authorship contribution statement

Amanda dos Santos Lima: Formal analysis, Methodology, Writing – original draft. **Luciana Azevedo; Nora Pap and Daniel Granato:** Conceptualization, Writing – original draft, Methodology, Supervision, Data curation, Funding acquisition. **Vinicius G. Maltarollo:** Formal analysis, Writing – original draft. **Lucas Cesar Pinheiro:** Formal analysis, Writing – original draft. **Frederico Augusto Ribeiro de Barros:** Formal analysis. **Mariana Araújo Vieira do Carmo and Thiago Mendanha Cruz**: Writing – original draft.

CRediT authorship contribution statement

Amanda dos Santos Lima: . **Vinicius G. Maltarollo:** Writing – original draft, Formal analysis. **Mariana Araújo Vieira do Carmo:** . **Lucas Cezar Pinheiro:** . **Thiago Mendanha Cruz:** . **Frederico Augusto Ribeiro de Barros:** . **Nora Pap:** Writing – original draft, Supervision, Methodology, Data curation, Conceptualization. **Daniel Granato:** Writing – original draft, Supervision, Methodology, Formal analysis, Data curation, Conceptualization. **Luciana Azevedo:** Writing – original draft, Supervision, Methodology, Formal analysis, Data curation, 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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