

Antioxidant Status, Antidiabetic Properties and Effects on Caco-2 Cells of Coloured and Non-Coloured Enriched Extracts of Sweet Cherry Fruits

Ana C. Gonçalves¹, M. Rodrigues^{1,2}, Adriana O. Santos¹, Gilberto Alves¹, Luís R. Silva^{1*}

¹ CICS-UBI – Health Sciences Research Centre, University of Beira Interior, 6201-506 Covilhã, Portugal; anacarolinagoncalves@sapo.pt

² UDI-IPG, Research Unit for Inland Development, Polytechnic Institute of Guarda, 6300-749 Guarda, Portugal; marciorodrigues@fcsaude.ubi.pt

¹ CICS-UBI – Health Sciences Research Centre, University of Beira Interior, 6201-506 Covilhã, Portugal; aos@ubi.pt

¹ CICS-UBI – Health Sciences Research Centre, University of Beira Interior, 6201-506 Covilhã, Portugal; gilberto@fcsaude.pt

¹ CICS-UBI – Health Sciences Research Centre, University of Beira Interior, 6201-506 Covilhã, Portugal; luisfarmacognosia@gmail.pt

* Correspondence: luisfarmacognosia@gmail.com; Tel.: +351 275 329 077

Abstract: This study aimed to compare three different extracts of *Saco* sweet cherry, namely non-coloured fraction, coloured fraction and total extract concerning phenolic composition, antioxidant and antidiabetic potential, erythrocytes' protection and effects on Caco-2 cells. A total of 22 phenolic compounds were identified by LC-DAD. Hydroxycinnamic acids were the most predominant in both non-coloured fraction and total extract, while cyanidin-3-*O*-rutinoside was the main anthocyanin found in the coloured fraction. The total extract was the most effective against DPPH, nitric oxide and superoxide radicals, and in the inhibition of α -glucosidase enzyme. Finally, the protective effect of the extracts to prevent oxidative damage in human erythrocytes was assessed. The coloured fraction revealed the best activity against hemoglobin oxidation and hemolysis. Regarding to Caco-2 cells, the coloured extract exhibited the most cytotoxic effects, while the total extract was the most efficient in protecting these cells against oxidative damage induced by *t*-BHP.

Keywords: Sweet cherry; Anthocyanins; Non-coloured phenolics; Antioxidant activity; Erythrocytes protection; Caco-2 cells.

1. Introduction

Phenolic compounds are widely distributed in nature and present strong antioxidant properties [1]. It is believed that their presence in the daily diet exerts a beneficial effect on human health, being associated with the decrease of oxidative stress-related disorders occurrence [1,2]. Furthermore, phenolic compounds reduce the rates of oxidative processes by acting as reducing agents, hydrogen donors, singlet oxygen quenchers and metal chelators, inhibiting the propagation of oxidizing chain reactions caused by free radicals and protecting the human body against oxidative damage [3–4].

In the last times, a special attention has been paid to the use of plants and fruits extracts in cosmetic, food and pharmaceutical industries due to their richness in phenolic compounds [5,6]. The extractions to obtain fractions rich in bioactive substances are preferentially carried out using water-alcohol solvent mixtures, often including ethanol or methanol, given their affinity with both lipophilic and hydrophilic bioactive molecules. Ethanol is the most commonly used solvent because it is economical, reusable and, unlike methanol, is non-toxic [5].

Extracts of sweet cherry (*Prunus avium* Linnaeus (L.)) have been subjected to several studies due to their properties as health promoters [7–9]. Therefore, sweet cherry fruits can be employed as food supplements, particularly in obese, diabetic and hypertensive subjects [10]. Although these fruits are preferably consumed in fresh, they also can be commercialized in processed products such as frozen,

canned, wine or concentrate juices, jellies, jams and dried forms [2]. They are rich in several non-coloured (chlorogenic acids, flavan-3-ols and flavonols) and coloured (mainly cyanidin-3-O-rutinoside) phenolic compounds, whose levels are regulated by genotype, fruit maturity, climatic conditions and storage conditions [6,7].

The largest European cherry-producing countries are Poland, Spain, Italy, Greece, Hungary and Germany [11]. In Portugal are produced around 15 thousand tons of cherries per year. Most of them are collected from Fundão region, being *Saco* one of the most important and oldest cultivars [7]. Previous works with *Saco* proved their large array of health benefits, namely antioxidant effects [7,6,9], anticancer activity against human cancer cells from colon (HT-29 and HCT-15) and stomach (MKN45) [6,9], antidiabetic properties and capacity to confer protection to human erythrocytes against hemoglobin oxidation and hemolysis [7].

Bearing in mind the above-mentioned reasons, the aim of this work was to improve the knowledge on the phenolic profile of total extract, and coloured and non-coloured fractions of *Saco* sweet cherry from Fundão region (Portugal). Furthermore, and knowing that coloured and non-coloured compounds can interact with each other in synergistic, additive and antagonistic ways, we also evaluated their antioxidant potential against 1,1-diphenyl-2-picrylhydrazyl (DPPH), nitric oxide ($\cdot\text{NO}$) and superoxide ($\text{O}_2\cdot^-$) radicals. The capacity of the extracts to inhibit α -glucosidase enzyme was also determined, as well as the protection afforded against induced oxidative damage in human erythrocytes. Additionally, the cytotoxic properties of the extracts were assessed for the first time against human colon carcinoma cells (Caco-2) under quiescent conditions concerning their antiproliferative activity, and toxicology responses when subjected to oxidative stress induced by tert-butyl hydroperoxide (*t*-BHP).

2. Materials and Methods

2.1. Chemicals and reagents

All chemicals used were of analytical grade. Cyanidin-3-O-glucoside, cyanidin-3-O-rutinoside, pelargonidin-3-O-rutinoside and peonidin-3-O-rutinoside were from Extrasynthese (Genay, France). The other phenolics and 1,1-diphenyl-2-picrylhydrazyl (DPPH $\cdot$), β -nicotinamide adenine dinucleotide (NADH), phenazine methosulfate (PMS), nitrotetrazolium blue chloride (NBT), α -glucosidase from *Saccharomyces cerevisiae* (type I, lyophilized powder), trypan blue, 2,2'-azobis (2-ethylpropionamidine) dihydrochloride (AAPH), *t*-BHP, high-glucose Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum (FBS), antibiotic (10000 U/mL penicillin, 10000 mg/mL streptomycin), trypsin-EDTA solution, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), dimethyl sulfoxide (DMSO), pyruvate, propidium iodide (PI), bovine serum albumin (BSA) and Ribonuclease A (RNase) were purchased from Sigma-Aldrich (St. Louis, MO, USA). N-(1-naphthyl)ethylenediamine dihydrochloride, sulfanilamide, 4-nitrophenyl-alpha-D-glucopyranoside (pNPG) and sodium nitroprusside dihydrate (SNP) were obtained from Alfa Aesar (Karlsruhe, Germany). Methanol and acetonitrile were acquired from Fisher Chemical (Leicestershire, United Kingdom). Water was deionized using a Milli-Q water purification system (Millipore Ibérica, S.A.U., Madrid). Human epithelial gastric adenocarcinoma (Caco-2) was from American Type Culture Collection (ATCC; Manassas, VA, USA).

2.2. Cherry samples

One kg of *Saco* sweet cherry cultivar was collected by hand from an orchard located in Fundão region (Portugal) at the same stage of ripeness, during June of 2016. The cherries were transported to the laboratory of Health Sciences Research Centre (CICS) of the University of Beira Interior (Covilhã, Portugal). The pits were removed, and the pulp was immediately frozen with liquid nitrogen and maintained at -20 °C. Then, the pulp was lyophilized, powdered (mean particle size lower than 910 μm) and separated into three aliquots and used for the preparation of the extracts.

2.3. Phenolic compounds

2.3.1. Extraction

Phenolic compounds of *Saco* samples were extracted according to a method previously reported by our research group [7]. A total of three extracts were prepared, designed as (i) non-coloured fraction, (ii) coloured fraction and (iii) total extract. The extraction yields of non-coloured fraction, coloured fraction and total extract were $8.5 \pm 0.02\%$, $3.6 \pm 0.005\%$ and $13.0 \pm 0.01\%$, respectively.

2.3.2. LC-DAD analysis

Twenty microlitres of each sample were analysed on a LC model Agilent 1260 system (Agilent, Santa Clara, California, USA) using a Nucleosil® 100-5 C18 column (25.0 cm $\times$ 0.46 cm; 5 μ m particle size waters; Macherey-Nagel, Düren, Germany), based on a previously published method [7]. Detection was achieved with an Agilent 1260 Infinity DAD using the ChemStation software supplied by Agilent Technologies (Waldbronn, Germany). Non-coloured fraction was re-dissolved with 4 mL of methanol, while coloured fraction was dissolved with 20 mL of acidified water, pH 3.0 using a membrane filter (0.45 μ m). The total extract was again eluted by SPE column to separate the non-coloured and coloured phenolics (anthocyanins) for identification and quantification.

The detection and quantification of anthocyanins and non-coloured phenolic compounds were performed according to Gonçalves et al. [7]. The compounds of each extract were identified by comparing their retention times and UV–VIS spectra in the 200–600 nm range with the library of spectra previously compiled by the authors. Anthocyanins were detected at 500 nm. Flavan-3-ols and hydroxybenzoic acids were detected at 280 nm, hydroxycinnamic acids at 320 nm and flavonols at 350 nm. The compounds unknown 1-3 were quantified as cyanidin-3-*O*-rutinoside. The hydroxybenzoic acid derivative 1 was quantified as *p*-hydroxybenzoic acid. The 3-*O*-caffeoylquinic acid and hydroxycinnamic acid derivatives 1 and 2 were quantified as 5-*O*-caffeoylquinic acid. *p*-Coumaric acid derivatives 1-5 were quantified as *p*-coumaric acid.

2.4. Biological evaluation

The evaluation of the biological potential of the non-coloured fraction, coloured fraction and total extract was performed spectrophotometrically by in vitro microassays using 96-well plates. The absorbances were measured in a microplate reader Bio-Rad Xmark spectrophotometer.

2.4.1. Antioxidant activity

2.4.1.1. DPPH

The capacity of non-coloured and coloured fractions and total extract to act as free radical scavengers against DPPH• was evaluated as previously reported [7].

2.4.1.2. Nitric oxide

The activity of the dried extracts against •NO was determined as previously described by Gonçalves et al. [7].

2.4.1.3. Superoxide radical

The effect of cherry extracts on O₂^{•-}-induced reduction of NBT was monitored at 562 nm. O₂^{•-} were generated by the PMS-NADH-O₂ system, as previously reported [12].

2.4.2. α -Glucosidase inhibitory activity

The α -glucosidase inhibitory activity was determined at 405 nm, based on Ellman's method, as previously described by Silva and Teixeira [12].

2.4.3. Protective effect in human erythrocytes against oxidative damage

Venous human blood was collected from randomized patients of Cova da Beira Hospital Centre (Covilhã), by antecubital venipuncture into K₃EDTA vacuum tubes. Erythrocytes were isolated based on the procedure previously described [13].

2.4.3.1. Inhibition of hemoglobin oxidation

The inhibition of hemoglobin (Hb) oxidation was evaluated by monitoring the effects of the three *Saco* extracts on the formation of methemoglobin, after the reaction of oxyhemoglobin with peroxy radicals (ROO[•]) generated by AAPH [7,13,14]. The absorbance was read at 630 nm. Quercetin was used as positive control. Three experiments were performed in triplicate for each extract.

2.4.3.2. Inhibition of hemolysis

ROO[•]s' were generated by AAPH and the prevention of ROO[•]-induced hemolysis of human erythrocytes was evaluated by monitoring the release of Hb after membrane disruption caused by the hemolytic process, according to the procedure described by Chisté et al. [13] and Gonçalves et al. [7]. The absorbance was obtained at 540 nm. Quercetin was used as positive control. Three experiments were performed in triplicate for each extract.

2.5. Cell culture conditions and treatments

Caco-2 cells were maintained in DMEM supplemented with 10% FBS and 2% of Pen/Strep. Cells were grown in 75 cm² culture flasks at 37 °C in a humidified air incubator with 5% CO₂. Once the cells reached 90-95% of confluence, they were washed twice with 10 ml of PBS and detached by gentle trypsinization (5 mL of trypsin-EDTA), and, before the experiments, viable cells were counted and suitably diluted in the adequate complete culture medium (25 000 cells per mL). These cell culture conditions and procedures were common through all assays. For the several assays, cells were used cells between passages 44 and 60. After the trypsinization and count of the cells, 200 μ L of the prepared cellular suspension (25 000 cells/mL) was seeded in 96-well plates and incubated for one day before carrying out the viability assays. Five concentrations in the range of 50-800 μ g/mL of each extract were dissolved in medium containing 0.5% (v/v) DMSO. The final concentration of DMSO did not affect cellular viability (data not shown).

2.5.1. Cytoprotection assay

Preliminary assays were done to determine the appropriate concentration and exposure time to *t*-BHP in order to evaluate the activity of the cherry extracts (data not shown). The *t*-BHP dilutions were prepared with concentrations that ranged from 0.25 to 4 mM and cells were exposed for 2, 4 and 6 h. By the analysis of the obtained results, and to achieve a suitable viability decrease, the selected exposure conditions were a time of 6 h with *t*-BHP at 1 mM. Cells were seeded under the same conditions previously described, with and without *t*-BHP co-incubation. After cells incubation during 24 h, the medium was removed, and cells were treated with the extracts during 24h, then the *t*-BHP was added to each well plate for 6 h. The difference of the assay without incubation is that after 24h of incubation with extracts, the medium was completely removed, and 1 mM of *t*-BHP was added to each well for 6 h. Finally, the MTT and lactate dehydrogenase (LDH) assays and the cell cycle distribution were carried out to evaluate the effect of the extracts against the induced toxicity.

2.5.2. MTT cell proliferation assay

To determine the effect of extracts on Caco-2 cells, viability was assessed by MTT assay after 24 h of exposure of each extract at different concentrations. Then, the medium was removed, and each well was washed with 200 μ L of PBS. The metabolic activity of cells was evaluated by their capacity to reduce the yellow MTT (0.5 mg/mL in the appropriate serum-free medium) to blue formazan product by 4 h of incubation at 37 °C. Then, the medium containing MTT was removed and the formazan crystals were dissolved in DMSO. The absorbance was read at 570 nm using a microplate reader Bio-Rad Xmark spectrophotometer. Cell proliferation values were expressed as percentages from the relative absorbance measured in the treated wells versus (vs) control wells [12,15]. A total of six independent experiments per extract were performed. Untreated cells were used as control.

2.5.3. LDH assay

The release of the stable cytosolic enzyme LDH into the media was spectrophotometrically determined at 340 nm, based on the conversion of pyruvate to lactate by LDH, using NADH as a cofactor [12]. The reaction mixture was composed of the extracts, NADH and pyruvate. All solutions were prepared in PBS (pH 7.4). The medium of each well plate treated with the extracts was collected after 24 h of cellular exposure and the LDH was evaluated. A total of six independent experiments per extract were performed. Untreated cells were used as control.

2.5.4. Cell cycle distribution analysis

The analysis of cell cycle distribution of cells was determined through PI staining of fixed and permeabilized cells. Briefly, 2.4 mL of Caco-2 cells were seeded in 12 well-plates (cell density of 25 000 cells/mL) in complete culture medium. After 24 h, they were treated with 200, 400 and 800 μ g/mL of each extract. Untreated cells were used as negative control. At the end of 24 h of incubation, each well was washed with PBS and the cells were harvested by trypsin treatment. The resulting cell suspension was kept on ice, pelleted by centrifugation, and resuspended in 450 μ L of a cold solution of 0.5% BSA in PBS. The resulting cell suspension was kept on ice and then fixed by gently adding ice-cold 70% ethanol (-20 °C) with simultaneous vortexing. After 1 day at -20 °C, fixed cells were washed twice with PBS and resuspended in a solution of PI prepared in PBS/BSA 0.5% (50 μ g/mL), passed through cell strainer filters (40 μ m nylon, Falcon®) and sequentially incubated with RNase at a final concentration of 7.1 μ g/mL (stock solution in 50% glycerol, 10 mM Tris-HCl, pH 8) for 15 min in the dark. Stained cells were then acquired at a BD Bio-sciences FACSCalibur flow cytometer (San Jose, CA, USA). Data were analysed using CellQuest™ Pro Software, version 5.1.1. First, singlets were selected by creating a region on the FL3-Width/FL3-Area contour plot. Then, gated singlet events were plotted on the FSC-Height/FL3-Area contour plots for correlation of size with PI-staining.

2.6. Statistical analysis of results

All data were recorded as mean $\pm$ standard deviation of triplicate determinations. The LC-DAD statistical phenolic comparison was made using the two-way ANOVA and the Bonferroni test. About to the biological potential, the statistical comparison was assured by the one-way ANOVA, and the means were classified by Tukey's test at a 95% level of significance. To determine the correlation between the antioxidant activity methods and the contribution of the total phenols, Pearson's correlation coefficients were calculated. Relatively to the cellular based assays, data from different groups were compared by two-way ANOVA followed by Dunnett's test (LDH and MTT assays) as *post-hoc* test. Values of $P < 0.05$ were considered statistically significant. All analyses were performed using Graph Pad Prism Version 6.01 (San Diego, CA, USA).

3. Results and Discussion

3.1. Phenolic profile

The LC-DAD analysis allowed the identification and quantification of a total of 22 phenolic compounds, including 2 hydroxybenzoic acid derivatives, 14 hydroxycinnamic acids, 1 flavonol and 5 anthocyanins (Tables 1 and 2). All of these compounds were previously described in *Saco* sweet cherry from Fundão (Portugal) [6,7]. As expected, the non-coloured phenolics were only found in the total extract and non-coloured fraction. Nevertheless, both extracts showed different quantitative composition (Table 1). The total contents of the non-coloured phenolics in the total extract and non-coloured fraction were 11069.7 and 15220.9 µg/g of dried extract, respectively (Table 1).

Table 1. Non-coloured phenolic contents of *Saco* sweet cherry extracts (µg/g of dried extract).

Non-coloured phenolics	Total extract	Non-coloured fraction
Hydroxybenzoic acid derivative 1	1337.85 ± 68.16	1839.54 ± 5.09 ^a
Hydroxycinnamic acid derivative 1	494.32 ± 51.66	679.69 ± 71.03
Hydroxycinnamic acid derivative 2	143.45 ± 21.30	197.24 ± 29.28
Hydroxybenzoic acid derivative 2	25.08 ± 0.92	34.48 ± 1.27
3- <i>O</i> -Caffeoylquinic acid	1482.97 ± 54.15	2039.09 ± 74.45 ^a
<i>p</i> -Coumaric acid derivative 1	50.02 ± 0.55	68.78 ± 0.76
<i>p</i> -Coumaroylquinic acid	nq	nq
Hydroxycinnamic acid derivative 3	372.26 ± 35.99	511.86 ± 49.48
5- <i>O</i> -Caffeoylquinic acid	734.38 ± 44.86	1009.77 ± 61.68 ^a
Hydroxycinnamic acid derivative 4	2835.87 ± 143.08	3899.33 ± 196.73 ^a
Caffeic acid	1263.49 ± 98.92	1737.30 ± 136.01 ^a
<i>p</i> -Coumaric acid derivative 2	528.74 ± 19.83	727.02 ± 27.26
Hydroxycinnamic acid derivative 5	704.96 ± 97.52	969.31 ± 134.08 ^a
Hydroxycinnamic acid derivative 6	196.75 ± 16.19	270.53 ± 22.26
<i>p</i> -Coumaric acid	21.07 ± 1.64	28.96 ± 2.26
Hydroxycinnamic acid derivative 7	666.97 ± 67.02	917.089 ± 92.15 ^a
Hydroxycinnamic acid derivative 8	175.97 ± 16.59	241.95 ± 22.80
Quercetin-3- <i>O</i> -glucoside	nq	nq
Kaempferol-3- <i>O</i> -rutinoside	nq	nq
Quercetin	35.58 ± 3.73	48.93 ± 5.13
Σ	11069.73	15220.88

Values are expressed as mean ± standard deviation of three assays. Σ, sum of the determined non-coloured phenolics and anthocyanins, respectively; nd; not detectable; nq, not quantified. ^aSignificant result ($P < 0.05$) is indicated as *vs* total extract.

In respect to phenolic acids and derivatives, they corresponded to 69.8% and 99.7% of the total phenolic compounds determined in total extract and non-coloured fraction, respectively (Table 1). The main compounds were hydroxycinnamic acid derivative 4 and 3-*O*-caffeoylquinic acid, representing 17.9% and 9.4% in the total extract, and 25.6% and 13.4% in the non-coloured fraction, respectively (Table 1). These results are in agreement with previous works, where the authors reported that the major compounds found in sweet cherries are hydroxycinnamic acids, mainly the 3-*O*-caffeoylquinic acid being this one also the most abundant compound found in *Saco* [6,7,9].

Additionally, one flavonol was detected and identified as quercetin, corresponding to 0.23% and 0.32% of total phenolic content in total extract and non-coloured fraction, respectively. No flavan-3-ols (e.g. catechins) were detected. This fact was not surprising to occur, given that Gonçalves et al.

[16] already have observed that catechin levels decreased in *Saco* cultivar during storage at room temperature (15 ± 5 °C) for 6 days.

In respect to anthocyanins, the analysis by LC-DAD led to the identification of three anthocyanins, being quantified a total of five compounds (Table 2). All of these coloured compounds were previously reported in *Saco* sweet cherry [6,7].

The total amount of anthocyanins found in the total extract and coloured fraction were 4740.7 and 19214.5 µg/g of dried extract, respectively (Table 2). Despite the different amounts of each anthocyanin observed, both extracts revealed a similar profile, being the unique difference related to the presence of two unknown anthocyanins detected only in the total extract (Table 2). Cyanidin-3-*O*-rutinoside was the main one quantified in both extracts, representing 24.5% and 81.5% of the total compounds for the total extract and coloured fraction, respectively, followed by cyanidin-3-*O*-glucoside. These data are in agreement with other previously reported works [6,7,10].

Comparatively with other cherries, tart cherries are richer in cyanidin-3-*O*-glucosylrutinoside (357.7 µg/g of dried extract), followed by cyanidin-3-*O*-rutinoside (226.1 µg/g of dried extract) and quercetin than sweet cherries (292.6 µg/g of dried extract) [17].

Table 2. Anthocyanin contents of *Saco* sweet cherry extracts (µg/g of dried extract).

Anthocyanins	Total extract	Coloured fraction
Unknown 1	2.99 ± 0.24	nd
Cyanidin-3- <i>O</i> -glucoside	193.48 ± 0.54	3427.93 ± 4.39 ^a
Cyanidin-3- <i>O</i> -rutinoside	3865.64 ± 2.95	15656.18 ± 25.71 ^a
Unknown 2	341.16 ± 2.82	nq
Pelargonidin-3- <i>O</i> -rutinoside	337.464 ± 20.19	130.39 ± 1.22 ^a
Peonidin-3- <i>O</i> -rutinoside	nq	nd
Σ	4740.73	19214.50

Values are expressed as mean ± standard deviation of three assays. Σ, sum of the determined non-coloured phenolics and anthocyanins, respectively; nd; not detectable; nq, not quantified. ^aSignificant result ($P < 0.05$) is indicated as *vs* total extract.

3.2. Antioxidant activity

Reactive species, including •NO, O₂• and ROO•, are products of normal cellular metabolism, playing crucial roles in signal transduction pathways, growth regulation, gene expression and immune responses [4]. Normally, their production in the human body is balanced by antioxidants; however, when they are overproduced, they cause damage in DNA, lipids and proteins, potentiating many human diseases, including cancer, diabetes, necrosis, neurological disorders and cardiovascular illnesses [1].

DPPH assay is widely used to determine the antioxidant activity of single compounds and plant extracts. All extracts studied in this work revealed to be able to scavenge DPPH radical in a concentration-dependent effect. The total extract was the most active ($IC_{50} = 21.88 \pm 0.32$ µg/mL), followed by the coloured and non-coloured fractions ($IC_{50} = 31.39 \pm 0.60$ and 210.86 ± 0.86 µg/mL, respectively). However, all extracts were less active than ascorbic acid control ($IC_{50} = 4.57 \pm 0.16$ µg/mL) (Fig. 1A and Table 3). Since phenolics are good hydrogen and electron donors and interact with each other rising the antioxidant ability, it was expected that the total extract, which has both coloured and non-coloured compounds, would be the most active [18].

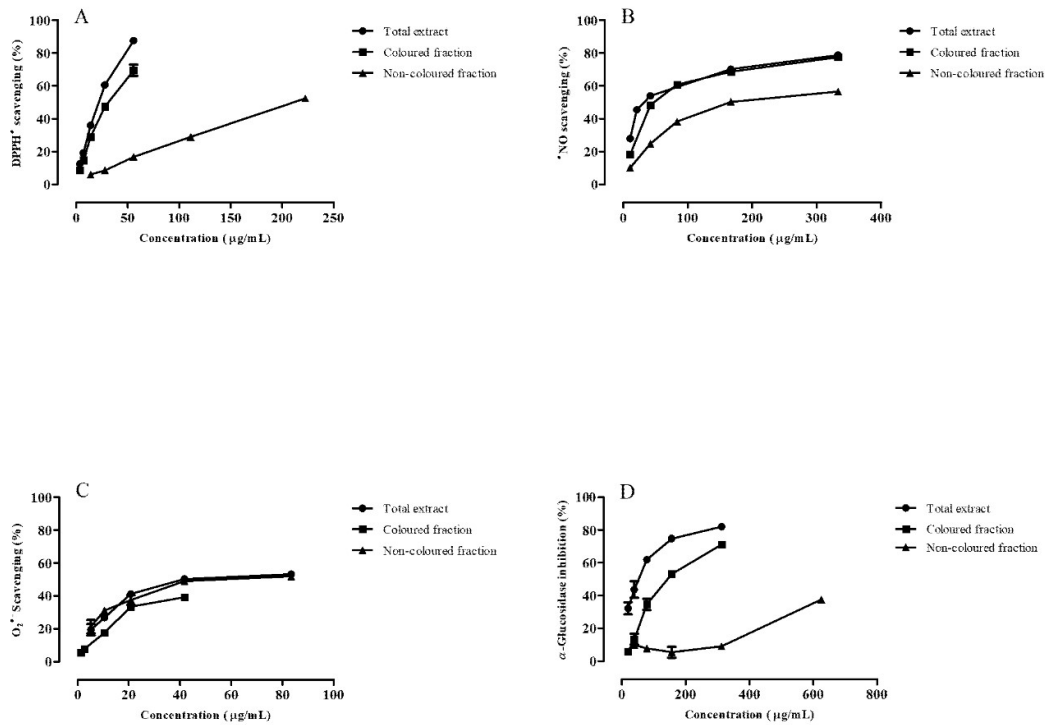


Figure 1. Antioxidant activity against (A) DPPH[•], (B) $\cdot$ NO and (C) O₂^{•-} radicals, and (D) α -glucosidase inhibition activity of *Saco* sweet cherry extracts.

Table 3. IC₂₅ and IC₅₀ (µg/mL) values found in the antioxidant activity, α-glucosidase, hemoglobin oxidation and hemolysis assays of *Saco* sweet cherry extracts.

Extract	DPPH•	•NO	O2•-	α-Glucosidase	Hemoglobin oxidation	Hemolysis
Total extract	21.88 ± 0.32	33.72 ± 0.89	41.68 ± 0.72	53.15 ± 1.32	34.29 ± 0.88	28.71 ± 0.73
Coloured fraction	31.39 ± 0.60 ^a	47.44 ± 0.67 ^a	16.58 ± 0.27 (IC ₂₅)	142.02 ± 1.17 ^a	33.86 ± 0.70	9.44 ± 0.48
Non-coloured fraction	210.86 ± 0.86 ^{a,b}	167.96 ± 0.92 ^{a,b}	69.40 ± 1.22 ^{a,b}	456.19 ± 3.74 (IC ₂₅)	155.13 ± 1.45 ^{a,b}	48.31 ± 1.07 ^{a,b}

Values are expressed as mean ± standard deviation of three assays.

^a Significant result (*P* < 0.05) is indicated as *vs* total extract.

^b *P* < 0.05 is indicated as *vs* coloured fraction.

The result obtained from total extract is in accordance with our previous work relative to *Saco* sweet cherry DPPH scavenging ability ($IC_{50} = 16.24 \pm 0.46 \mu\text{g/mL}$) [7]. Considering other red-fruit extracts, the three extracts showed more potential than anthocyanin-enriched fraction of *Romina* strawberry fruits ($IC_{50} = 1590 \pm 3.54 \mu\text{g/mL}$), while the coloured fraction of *Saco* proved to have similar activity to whole methanolic extract of strawberries ($IC_{50} = 30.29 \pm 0.18 \mu\text{g/mL}$) [19]. In addition, our total and coloured extracts revealed to be more active than crude, petroleum ether, chloroform, ethyl acetate and butanol fractions of myrtle fruits (*Myrtus communis*) ($IC_{50} = 130.44 \pm 12.1$, 108.3 ± 10.2 , 111.14 ± 11.1 , 85.6 ± 5.7 and $84.42 \pm 1.8 \mu\text{g/mL}$, respectively) [20].

The antioxidant capacity of the extracts also was evaluated against $\bullet\text{NO}$ and $\text{O}_2\bullet^-$. Both radicals are present in human body. While NO is normally produced in the organism as a second messenger and as a part of immune response, its reaction with $\text{O}_2\bullet^-$ leads to the production of more toxic free radical species, such as peroxynitrite and $\text{ROO}\bullet$, increasing deleterious effects in cells [1]. Presently, there is much evidence about the beneficial effects of phenolic-rich extracts on $\bullet\text{NO}$ and $\text{O}_2\bullet^-$ species' elimination [7,12].

In this study, all *Saco* extracts displayed a great capacity to capture $\bullet\text{NO}$ in a concentration-dependent manner (Fig. 1B and Table 3). The total extract was the most active ($IC_{50} = 33.72 \pm 0.89 \mu\text{g/mL}$), followed by the coloured and non-coloured fractions ($IC_{50} = 47.44 \pm 0.67$ and $167.96 \pm 0.92 \mu\text{g/mL}$, respectively) (Fig. 1B and Table 3). All extracts showed better ability to scavenge $\bullet\text{NO}$ than ascorbic acid control ($IC_{50} = 179.69 \pm 2.06 \mu\text{g/mL}$). The total extract was the most effective, given the fact that the natural combination of both non-coloured phenolics and anthocyanins increases the capture of radical species through electron delocalization after hydrogen donation by the hydroxyl (OH) groups, diminishing the concentration of these undesirable species [4].

Indeed, the capacity of sweet cherries to scavenge $\bullet\text{NO}$ was previously reported by Jacob et al. [21]. The authors verified a decrease on NO levels in ten healthy women, 3 h after consumption of 280 g of *Bing* sweet cherries. Relatively to other red-fruits, our extracts showed better activity than ethanolic ($IC_{50} = 1600 \pm 0.03 \mu\text{g/mL}$) and aqueous extracts ($IC_{50} = 1500 \pm 0.02 \mu\text{g/mL}$) of blueberry fruits (*Vaccinium corymbosum* L.) [1]. In addition, both total extract and coloured fraction proved to be more active than ethanolic strawberry extracts ($IC_{50} = 118 \pm 45.2 \mu\text{g/mL}$) [22]. In comparison with ethanolic grape extracts, grapes displayed more ability in neutralizing $\bullet\text{NO}$ ($IC_{50} = 5.1 \pm 0.2 \mu\text{g/mL}$) [22].

In respect to the ability of *Saco* extracts to scavenge $\text{O}_2\bullet^-$, all of them showed activity in a concentration-dependent effect. The results obtained from total and non-coloured extracts were $IC_{50} = 41.68 \pm 0.72$ and $69.40 \pm 1.22 \mu\text{g/mL}$, respectively (Fig. 1C and Table 3). The coloured fraction revealed the weaker activity ($IC_{25} = 16.58 \pm 0.27 \mu\text{g/mL}$). Even so, all the three extracts demonstrated to be less active than ascorbic acid control ($IC_{50} = 28.87 \pm 0.38 \mu\text{g/mL}$). The total extract was the most active. The presence of both coloured and non-coloured phenolics, increases and diversifies their interactions, and intensifies the antioxidant activity [1].

The scavenging ability of sweet cherry fruits against $\text{O}_2\bullet^-$ radical was already demonstrated by Prior et al [23]. In respect to other red fruits, our total extract exhibited similar activity to vitamin C rich fraction of bilberry fruits (*Vaccinium myrtillus* L.) ($IC_{50} = 49 \mu\text{g/mL}$), but less efficiency than their flavonoid ($IC_{50} = 21 \mu\text{g/mL}$) and phenolic acid ($IC_{50} = 15 \mu\text{g/mL}$) rich fractions [24]. Moreover, our total extract and non-coloured fraction proved to have higher ability to scavenge these species when compared with ethanolic extracts of strawberry fruits ($IC_{50} = 72.5 \mu\text{g/mL}$), but less activity than those of acetone ($IC_{50} = 9.7 \mu\text{g/mL}$). Furthermore, our total extract showed similar activity to strawberry ethyl acetate extracts ($IC_{50} = 40.1 \mu\text{g/mL}$) [25].

Our work is another report that supports the strong connection between the antioxidant capacity and the phenolic content. It is well-known that phenolic compounds can easily capture free radical species and chelate metal ions due to their acid moiety and OH groups [18]. The phenolic OH groups on B-ring easily donate hydrogen atoms or electron to radicals, stabilizing them [1]. Additionally, the OH groups present in the unsaturated C-ring and the double bond on this ring also enhance the antioxidant capacity [4]. Following this same order of context, it is important to emphasize the presence of cyanidin-3-O-rutinoside in both total extract and coloured fraction. This anthocyanin presents several OH groups in its structure, being one of the phenolics more responsible for the

biological activity of the fruits [26]. Furthermore, both coloured and non-coloured phenolics interact with each other, through additive and synergistic combinations, conferring to sweet cherries great antioxidant effects [1,18].

In fact, by Pearson's test, there were found positive correlations between the antioxidant tests and the total amount of phenolic compounds. High correlations ($r > 0.9013$) were obtained between the majority of the non-coloured phenolics, anthocyanins and antioxidant activities measured by DPPH•, •NO and O2• assays. Even so, we found a negative correlation between O2• scavenging test, cyanidin-3-*O*-glucoside ($r = -0.9818$) and cyanidin-3-*O*-rutinoside ($r = -0.9818$), and between DPPH• and •NO antioxidant assays, and pelargonidin-3-*O*-rutinoside ($r = -0.9241$ and -0.9660 , respectively). Finally, it is important to remember the possible presence of other non-determined reducing compounds, such as organic acids, carotenoids and volatile compounds, which may also contribute to increasing the antioxidant potential, and that were not determined in this work.

3.3. α -Glucosidase inhibitory activity

Diabetes mellitus is an epidemic metabolic disease, characterized by chronic hyperglycemia and glucose intolerance caused by defects in insulin hormone that affects million people worldwide [2]. One therapeutic approach is to restore blood glucose levels as close to normal as possible via inhibition of carbohydrate hydrolysing-enzymes, as α -glucosidase [27]. Over the years, many phenolic compounds present in plants have shown to be capable to inhibit the action of α -glucosidase, thereby delaying glucose uptake [7,27].

In our study, all *Saco* extracts tested proved to have the capacity to inhibit the α -glucosidase enzyme in a concentration-dependent effect. The total extract showed the most effectiveness ($IC_{50} = 53.15 \pm 1.32 \mu\text{g/mL}$), followed by the coloured ($IC_{50} = 142.02 \pm 1.17 \mu\text{g/mL}$) and non-coloured fractions (IC_{25} value of $456.19 \pm 3.74 \mu\text{g/mL}$) (Fig. 1D and Table 3). The obtained IC_{50} values were much lower than acarbose control ($IC_{50} = 389.89 \pm 4.01 \mu\text{g/mL}$), one of the therapeutic drugs most recommended to treat type 2 diabetes but whose use is limited since it causes gastrointestinal problems, as diarrhoea, flatulence and intestinal pain [7]. Both coloured and non-coloured phenolics interact among all and with the substrate of the enzyme and create bonds with this one, thus interfering with its action and consequently preventing the carbohydrate digestion, as well as protecting pancreatic β -cells from oxidative stress levels, contributing for their normal functioning [28].

The antidiabetic properties of sweet cherries are already known [7,29,30]. Lachin [30] reported that diabetic rats fed with 200 mg of cherry extract per kg of body weight for 30 days showed reduced blood glucose and urinary microalbumin levels, proving that the ingestion of these fruits can protect pancreatic β -cells and retard glucose absorption. Besides, Cao et al. [29] revealed that fractionated extracts of *Black Pearl* sweet cherry cultivar rich in anthocyanins, hydroxycinnamic acids and flavonols also demonstrated antidiabetic properties, by promoting cellular glucose consumption in HepG2 cells.

Comparatively with the antidiabetic potential of other fruit extracts, our total extract and coloured fraction exhibited a better capacity to inhibit α -glucosidase enzyme than dried crude acetone extracts of *Ficus lutea* and *Ficus sycomorus* ($IC_{50} = 290 \pm 111$ and $217 \pm 69 \mu\text{g/mL}$, respectively) [31].

The positive results obtained with *Saco* extracts to inhibit α -glucosidase action are largely due to their phenolic constituents. In these ones, the unsaturated C-ring, the linkage of the B-ring at the position 3, the OH substitution on the B-ring and the presence of either 3-OH and 4-CO groups enhance their ability to inhibit α -glucosidase [3].

In addition, they interact not only synergistically and additively between them and with other compounds, but also in both competitive and non-competitive ways with the substrate of the enzyme, increasing the antidiabetic potential of this fruit [3,7]. Also, phenolics can bond with digestive enzymes through hydrophobic association, inhibiting the action of them [32]. For these reasons, both coloured and non-coloured phenolic compounds can promote the insulin production and the proliferation of pancreatic β -cells and can interfere with glucose homeostasis by modifying

independent mechanisms and increasing insulin receptor-dependents (such as PPAR γ), thus exerting antidiabetic actions [33].

In our study, it is possible to state that non-coloured compounds have practically no significant and notorious effects on the inhibition of α -glucosidase, whilst anthocyanins showed great effectiveness. These results were expected, since previous studies reported that the coloured compounds are the main responsible for the antidiabetic capacities revealed by cherries, mainly due to their high antioxidant abilities (that are enhanced by the presence of the 3-OH group and the OH substitution on the B-ring in anthocyanins) that allow the protection of insulin mechanisms against oxidative damage and offer capacity to inhibit intestinal enzymes [3,28,34]. To reinforce this fact, the Pearson's test was performed and there was obtained a high correlation between the α -glucosidase inhibitory assay and the total amount of anthocyanins ($r = 0.9929$).

Additionally, the presence of rutinose in the 3-O-position of cyanidin, which already has many OH groups, reinforces this health-promoting property [28]. Indeed, the main coloured compound present in sweet cherries, that is cyanidin-3-O-rutinoside, already demonstrated to have a significant inhibitory effect in α -glucosidase activity, in a concentration-dependent manner, by competing with glucose for the binding site on sodium-dependent glucose transporter (SGLT) 1, and consequently delaying the absorption of glucose [3,35]. More recently, Adisakwattana et al. [35] reported that cyanidin and its glycosides also create covalent and/ or non-covalent interactions between their OH groups and the polar groups (amide, guanidine, peptide, amino and carboxyl groups) of amino acids in the active site of carbohydrate digestive enzymes, stopping their action. The same work also revealed that a single oral administration of cyanidin-3-O-rutinoside (100 and 300 mg per kg) significantly decreases plasma glucose levels in rats at 30, 60 and 90 min after maltose and sucrose administration. Furthermore, cyanidin-3-O-glucoside also promotes the release of insulin by pancreatic β -cells and glucose uptake by enhancing glucose transporter GLUT 4 [33].

Non-coloured compounds can also interfere with the activity of digestive enzymes and prevent insulin resistance events [34]. Particularly chlorogenic acids already showed to be able to increase GLUT 4 expression via the PI3K-independent pathway, as well as to suppress hepatic gluconeogenesis through the inhibition of glucose 6-phosphatase activity, and to inhibit Na⁺-dependent glucose transporters SGLT 1 and SGLT 2 [34]. Caffeic acid also showed ability to enhance glucose-stimulated insulin secretion in mice pancreatic islets at concentrations from 10^{-10} to 10^{-6} M and the expression of key insulin regulatory genes INS1, INS2, PDX1, INSR, IRS1, MAFB and GLUT2 [36].

3.4. Protective effects of *Saco* extracts against ROO• in human blood samples

Erythrocytes are highly susceptible to being attacked by reactive species due to their content in unsaturated fatty acids, oxygen, hemoglobin and in transition metals such as copper and iron [37]. In addition, they are more frequently exposed to oxygen than other body tissues and so they are more vulnerable to oxidative damage, causing the oxidation of lipids and proteins in the cell membrane, and thus inducing hemolysis [38]. Previous studies already have shown that phenolics present in plants offer therapeutic benefits against oxidative damage in erythrocytes [7,38,39].

Figure 2A shows the protective effects of *Saco* extracts to avoid hemoglobin oxidation in a concentration-dependent manner. To the best of our knowledge, this is the first study about the capacity of coloured- and non-coloured- rich fractions of sweet cherries to prevent hemoglobin oxidation. The coloured fraction and the total extract were the most active and displayed similar activity ($IC_{50} = 33.86 \pm 0.70$ and 34.29 ± 0.88 μ g/mL, respectively) (Fig. 2A and Table 3), however they were seven times less active than quercetin control ($IC_{50} = 4.38 \pm 0.42$ μ g/mL) analysed in the same conditions. The non-coloured fraction showed an IC_{50} value of 155.13 ± 1.45 μ g/mL (Fig. 2A and Table 3).

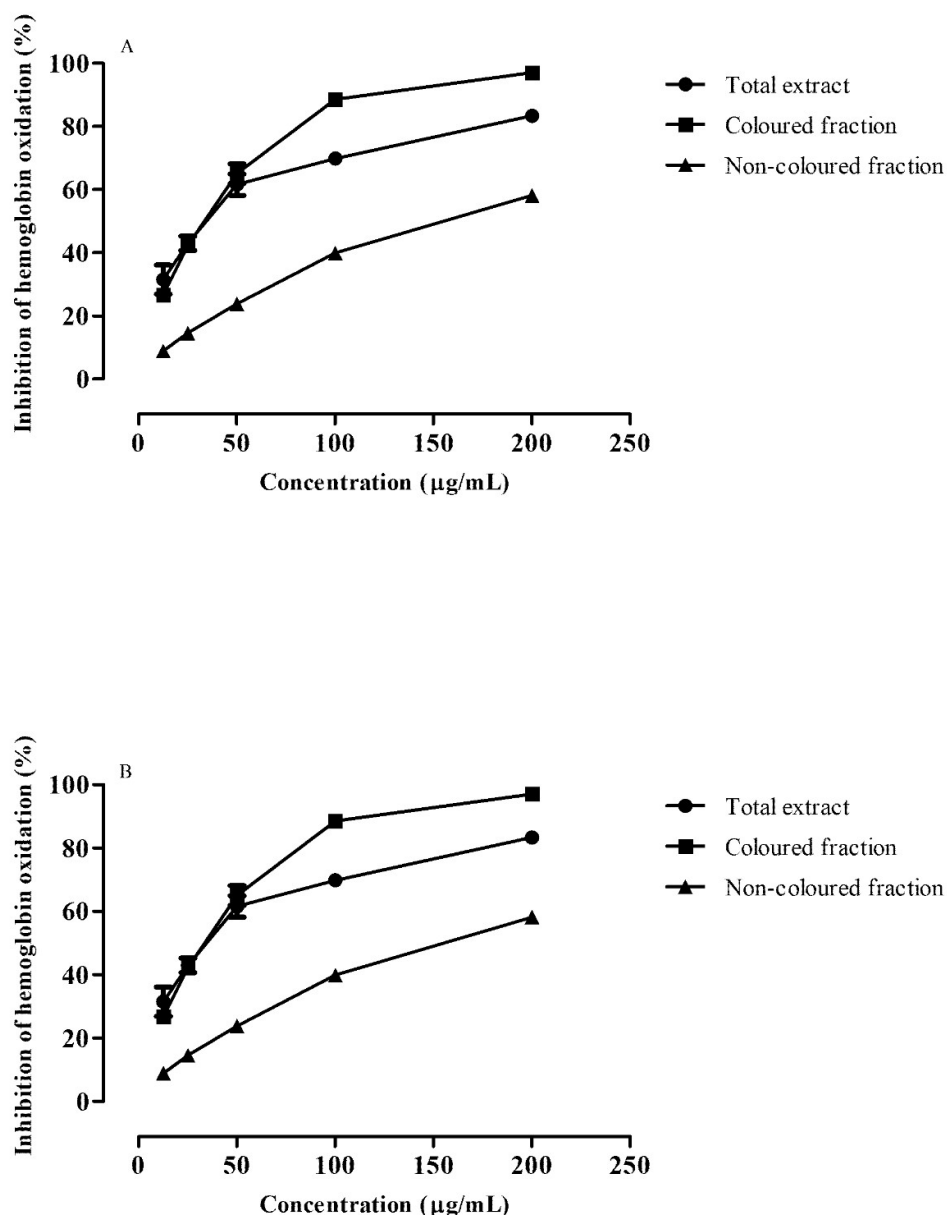


Figure 2. Inhibition of (A) hemoglobin oxidation and (B) hemolysis by *Saco* sweet cherry extracts.

As far as we know, few studies exist on the ability of fruit extracts to prevent the oxidation of hemoglobin. Previously, we have reported that the total extract of *Saco* possesses the capacity to prevent hemoglobin oxidation [7]. Hydrophilic extracts of murici fruits (*Byrsonima crassifolia*) also showed this property, revealing an IC_{50} of $271 \pm 44 \mu\text{g/mL}$, 10 being less active than *Saco* extracts.

The capacity of phenolic compounds to inhibit oxidative damage is closely associated with the strong antioxidant capacity exhibited by themselves [18]. Both coloured and non-coloured phenolics can penetrate into the erythrocytes' membrane due to their lipophilic character, causing a reduction in its fluidity and stability, and thus preventing the propagation of these harmful radicals [37]. The biological activity of the total extract and coloured fraction is enhanced by the presence of catechol rings and OH groups, principally in cyanidin-3-*O*-rutinoside, increasing the potential of both extracts [40], as well as the ability to inhibit the oxidation of hemoglobin by oxidizing the heme iron of erythrocytes [41]. In this way, they prevent enzymatic reactions and consequently oxidative events.

Another experiment was conducted in order to evaluate the capacity of *Saco* extracts to prevent hemolysis. The addition of APPH generated ROO[•] capable to attack the membrane of erythrocytes from the outside, promoting hemolysis [37]. As far as we know, this is the first report concerning lysis prevention by both coloured and non-coloured fractions of sweet cherry fruits. The coloured fraction ($IC_{50} = 9.44 \pm 0.48 \mu\text{g/mL}$) showed the best anti-hemolytic protection, followed by the total extract ($IC_{50} = 28.71 \pm 0.73 \mu\text{g/mL}$) and non-coloured fraction ($IC_{50} = 48.31 \pm 1.07 \mu\text{g/mL}$) (Fig. 2B and Table 3). All extracts exhibited less activity than quercetin control ($IC_{50} = 1.58 \pm 0.12 \mu\text{g/mL}$) (Fig. 2B and Table 3).

Saco total extract already proved to be able to prevent hemolysis [7]. Considering other fruit extracts, *Saco* extracts exhibited more potential to inhibit hemolysis than aqueous extracts of strawberry fruits (*Arbutus unedo* L.) ($IC_{50} = 430.00 \mu\text{g/mL}$) [26]. Furthermore, the coloured fraction displayed similar activity to methanolic extracts of grape fruits (*Ruby Cabernet*) ($IC_{50} = 11.62 \mu\text{g/mL}$) [39].

These results reinforce the fact that phenolic compounds, principally anthocyanins, can scavenge free radicals before they attack the membrane of erythrocytes, diminishing their concentration, and thereby preventing hemolysis [37]. These results were supported by another work concerning the anti-hemolytic effects of phenolics from honey, where it was also verified that they can capture free radicals, preventing lysis' incident [40].

3.5. Effect of sweet cherry extracts in Caco-2 cell viability

As referred above, it was already reported that sweet cherries have antidiabetic properties [7,29,30]. Hereupon, human colorectal adenocarcinoma Caco-2 cells were selected given two reasons: (i) they are a model of the intestinal epithelium, since, after differentiation, they form monolayers, that reproduce several characteristics of intestinal epithelial cells (e.g. the formation of microvilli at the apical cell surface, the tight junctions between cells, and the expression of brush-border proteins including digestive enzymes, transporters and receptors; and (ii) it is important to note that after consumption of cherries, their compounds directly contact with intestinal epithelium [15].

In a first step and given that phenolic compounds can exert a dose-dependent cytotoxicity; a preliminary experiment was conducted to determine the range of concentrations for which the exposure to each extract does not affect the cellular viability. As can be seen in Fig. 3, Caco-2 cells proved to be more sensitive to the coloured fraction when compared to the total extract and non-coloured fraction, exhibiting decreases concerning their cellular viability, that ranged from 93.11% (200 $\mu\text{g/mL}$), 73.66% (400 $\mu\text{g/mL}$), 37.90% (800 $\mu\text{g/mL}$), and an IC_{50} value of $667.84 \pm 2.46 \mu\text{g/mL}$. Additionally, and as expected, the more significant LDH response was also obtained using the coloured fraction, mainly for the higher concentrations, such as 200, 400 and 800 $\mu\text{g/mL}$ (with values of 116.5, 126.3 and 151.1%, respectively) (Fig. 3).

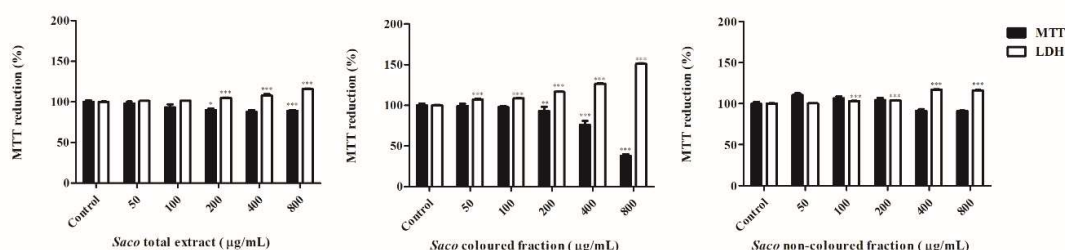


Figure 3. Effect of *Saco* extracts on Caco-2 cell lines viability after 24 h of exposure, assessed by MTT reduction and LDH leakage assays. Values show mean $\pm$ SEM of six independent assays performed in triplicate (* $P < 0.05$, ** $P < 0.01$ and *** $P < 0.0001$).

The obtained data suggest that the loss of mitochondrial activity happens prior to membrane's damage given that the results of MTT were more expressive than those of LDH, thus discarding a

necrotic process in the lowest concentrations ($\leq 200 \mu\text{g/mL}$) and its occurrence in the highest concentrations (400 and $800 \mu\text{g/mL}$), which is accompanied by an elevation of LDH in culture medium [42–44]. Indeed, phenolics can interfere in different steps of carcinogenesis (promotion, initiation and progression) acting as a blocking agent or as a cancer suppressor, inhibiting the metabolic action of the pre-carcinogens, and consequently, blocking the tumour initiation [44,45]. In our work, higher concentrations of phenolics ($> 200 \mu\text{g/mL}$) may induce DNA damage and necrosis in cancer cells [43,44].

The remarkable effect of the coloured extract is not surprising, taking into account that the anthocyanins promote cell death in cancer cells, triggering apoptosis through two major cell-intrinsic pathways: either by binding to the death receptors on the cell surface or by promoting the mitochondrial release of cytochrome C, inciting the depolarization of the mitochondrial membrane and the degradation of the poly[ADP-ribose] polymerase 1 [46]. This fact is supported by the positive correlation obtained by the total anthocyanin content and the inhibitory proliferation activity ($r = 0.6674$). These effects are also enhanced by their three OH groups on the β -ring, which are the main responsible for their biological activity [18]. Additionally, non-coloured phenolics also exhibit antiproliferative effects. Particularly, Yen et al. [47] verified that many phenolic compounds, including quercetin derivatives, caffeic, chlorogenic and *p*-coumaric acids, showed cytotoxicity and strong inhibitory effects on human cancer cell growth, once again due to their antioxidant capacities.

In order to further explore the cellular mechanism of growth inhibition by cherry extracts, a morphological assessment and an attempt of evaluation of cell cycle were performed. Apoptosis and necrosis represent two fundamental types of cell death. On the one hand, apoptosis causes several morphological and biochemical changes in cells that may take hours or days. From the morphological point of view, it is usually associated with cell shrinkage and bleb formation [47]. On the other hand, necrosis occurs suddenly, and it is characterized by mitochondrial and cellular swelling following by plasma membrane disruption [43]. This study was performed upon incubation with the cherry extracts using the higher concentrations (200, 400 and $800 \mu\text{g/mL}$), which were those that showed promising antiproliferative activity. The control cells positively marked with PI and gave a characteristic histogram on linear PI-fluorescence channel, but unfortunately, there was not possible to assess the cell cycle on treated cells because the number of events was not sufficient (data not shown) due to extensive cell death, as was apparent at observation the under the optical microscope (Figures 4 and 5). While the acquisition of controls was stopped when about 10000 singlet events were acquired, treated cells were acquired until no more cell suspension was left, and originated a much lower event count. To visualise simultaneously morphological information (size) and PI-staining intensity of events, data were plotted on FSC-height vs FL3-Area contour plots (Fig. 4). PI-stained and normal sized events decreased as the concentration increased [upright (UR) part of the plots, Fig. 4B, 4E, 4H for the total extract, 4C, 4F and 4I for the coloured fraction, and 4D, 4G and 4J for the non-coloured fraction, respectively]. Furthermore, in the highest tested concentrations of total extract and coloured fraction (400 and $800 \mu\text{g/mL}$), there was a significant increase in the number of debris with a size bigger than usual (upper left quadrant), which may be evidence of necrotic processes (Fig. 4E and 4H for the total extract, and 4F and 4I for the coloured fraction, respectively). Beyond that, and as expected the coloured fraction was the most cytotoxic, showing the lowest event number (Fig. 4I). This fact agrees with the previous result obtained through MTT and LDH assays concerning the coloured fraction (Fig. 3).

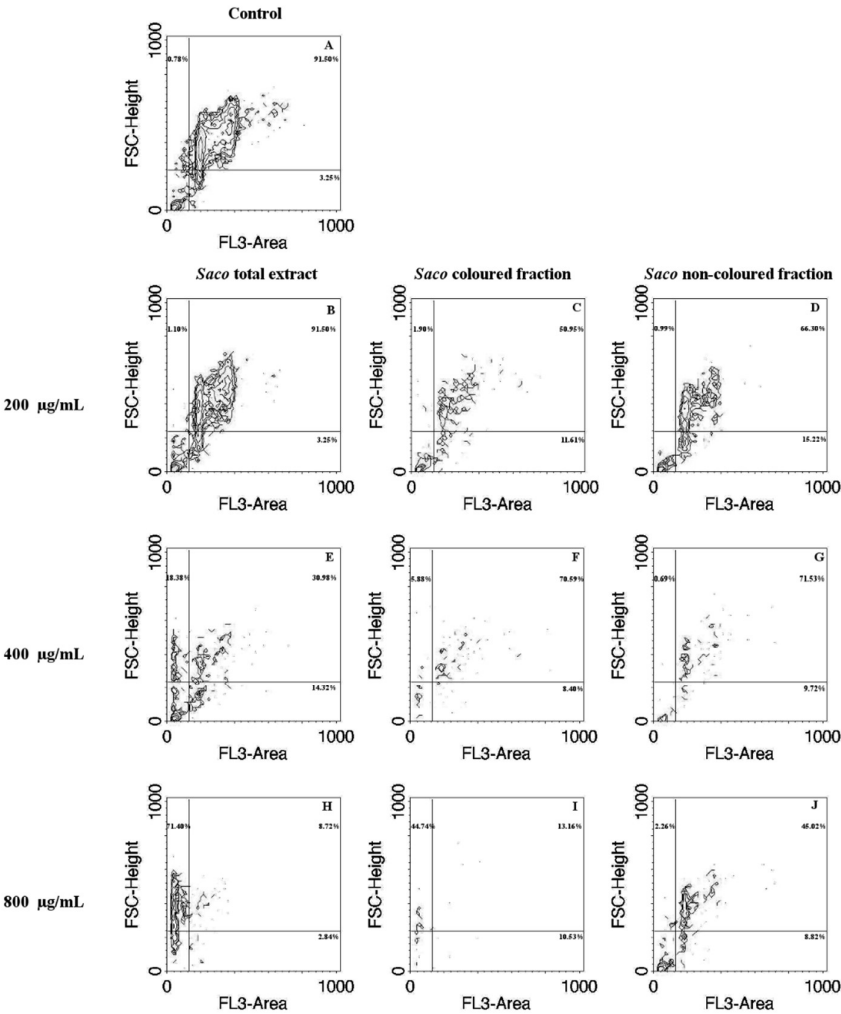


Figure 4. Percentage of cell survival after 24 h treatment with 200, 400 and 800 µg/mL of each extract in Caco-2 cell lines through PI flow cytometric assay. The control corresponds to untreated cells. The percentage of survival is the percentage of live cells as compared to the total number of events of both live and dead cells.

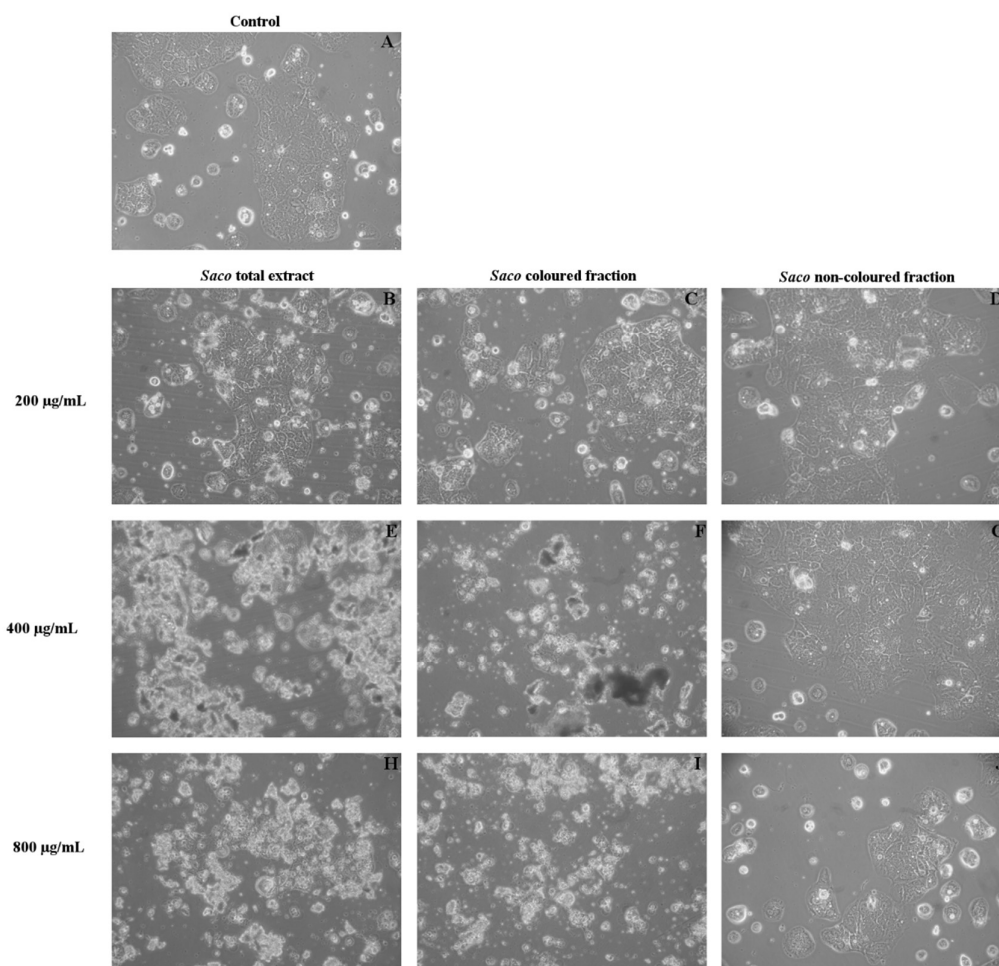


Figure 5. Morphological assessment of Caco-2 cells (control vs treatment, 24 h of incubation).

At the morphological observation of cultures under the microscope, it was observed a high number of debris (Fig. 5), that increased with the increase of concentration (Fig. 5H 5I and 5J). These data were to be expected because it was already mentioned that high concentrations of phenolics can induce acute toxicity effects on cancer cells. Therefore, this work is another evidence of the toxicity and pro-oxidant effects of higher levels of phenolic compounds, which are capable of affecting cell functions such as growth and differentiation, causing probably both apoptosis and necrotic events. Specifically, it was already documented that quercetin may induce an initial shock to the cells, resulting in necrosis, followed by a reorganization of the remaining viable cells that will undergo apoptosis after prolonged treatment [43]. Furthermore, Wang et al. [44], concerning the inhibitory effect of blueberry anthocyanin extracts on melanoma cells, verified that total apoptotic cells increased gradually at concentrations of 0–800 µg/mL in a dose-dependent manner, with necrosis occurring from the concentration of 300 µg/mL.

3.6. Cytoprotection assay

The second step was to evaluate the potential of each extract to protect the cells against the toxicity caused by *t*-BHP. *t*-BHP is an organic peroxide metabolized by cytochrome P450 widely used, being a better alternative than hydrogen peroxide in oxidation-induced stress studies since it creates more stable radical species, particularly toxic peroxy and alkoxyl radicals that affect cell integrity and form covalent bonds with cellular molecules, promoting the death of cells [48].

Cells were treated with different concentrations of each extract for 24 h prior to *t*-BHP exposure (1mM, 6 h). Cellular viability was again determined by MTT and LDH leakage assays. All the extracts

exerted a dose-dependent protective effect in the MTT reduction and LDH leakage assays (Fig. 6). Pre-treatment of Caco-2 cells with each extract was seen to significantly prevent reductions in cell viability. The total protection, when compared to stressed control cells, was almost achieved at the concentrations of 50 and 200 $\mu\text{g/mL}$ with and without co-incubation with *t*-BHP for the total extract and non-coloured fraction. While for the coloured fraction, this one was achieved at concentrations of 50 and 100 $\mu\text{g/mL}$ with and without co-incubation, respectively (Fig. 6). Relatively to the assay with co-incubation, there were observed increments of viability between 2.89 and 25.45% for the total extract, 4.98 and 14.46% for the non-coloured fraction, and between 1.73 and 17.22% for the coloured fraction (Fig. 6). Concerning the obtained data without co-incubation, it was observed lower values of protection, ranging from 25.00 to 33.58% for the total extract, 18.15 to 28.96% for the non-coloured fraction, and 16.90% to 27.02% for the coloured fraction (Fig. 6). This outcome indicates that the presence of phenolics in the medium, even outside the cells, also offers protection against cellular damage [44].

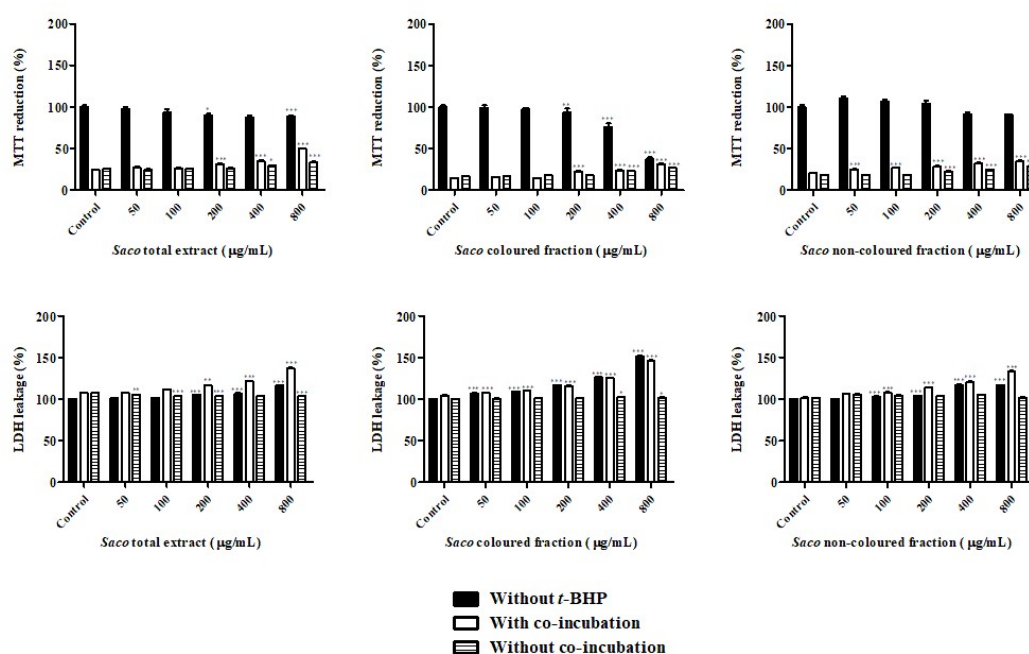


Figure 6. Caco-2 cellular viability assessed by MTT reduction and LDH leakage assays, after exposure to *Saco* extracts, with and without *t*-BHP-induced toxicity. Cells were pre-treated with each extract for 24 h. Insulted cells were further exposed to *t*-BHP (1 mM) for 6 h. Values show mean $\pm$ SEM of six independent assays performed in triplicate (* $P < 0.05$, ** $P < 0.01$ and *** $P < 0.0001$ compared to the respective controls).

In both assays, the highest protection was verified with the total extract at the concentration of 800 $\mu\text{g/mL}$, which is another support about the simultaneous interaction and synergy between coloured and non-coloured phenolic compounds [44].

Once again and given the oxidative-stress induced by *t*-BHP, it was verified an increase in LDH leakage, most notorious in the assay without co-incubation, revealing that the permanence of phenolics in the medium increases the protective effects on cells. Our results are in agreement with the work of Bedoya-Ramírez et al. [49], who reported that 500 $\mu\text{g/mL}$ of Colombian coffee can increase cell viability between 34 and 45% evaluated by MTT assay.

The phenolic composition of each extract is described in Tables 1 and 2. In fact, phenolics are the great responsible for the observed antioxidant protective effects. As we know, mitochondria are very susceptible to oxidative stress induced by oxygen species generated continuously in these organelles

in the course of oxidative stress [48]. Phenolics, essentially due to their hydroxyl and carboxyl groups exhibit promising and strong antioxidant activities being capable of capturing these radicals and chelating metals before they attack the mitochondrial membrane [42]. Particularly, 50 μ M of quercetin derivatives already showed similar protective effects at preventing Caco-2 damage induced by *t*-BHP [50].

4. Conclusions

Considering the current interest in cherries, the present study describes the phenolic constitution and health-promoting properties of three extracts from *Saco* sweet cherry. A total of 22 phenolic compounds, including 17 non-coloured phenolics and 5 anthocyanins, were identified, being hydroxycinnamic acids and cyanidin-3-*O*-rutinoside the main ones. In relation to biological potential, in a general way, the total extract proved to be the most active given the interactions established between both coloured and non-coloured phenolic compounds. Even so, all extracts revealed facility to scavenge free radical species and to inhibit the α -glucosidase intestinal enzyme. Concerning human erythrocytes protection, the coloured fraction was the most effective, mainly due to the presence of many OH groups in anthocyanins, which increases the capacity of scavenging ROO $\cdot$ species. On the other hand, and relatively to Caco-2 cell study, the highest tested concentrations of each extract showed ability to inhibit the proliferation of Caco-2 cells mainly due to their antioxidant properties. Additionally, both fractions and the total extract exerted cytoprotective effect against oxidative stress induced by *t*-BHP in Caco-2, improving cell viability. Once again, this protection is closely linked to the capacity of phenolics to pass through the cellular membrane and act as direct antioxidants, capturing free radicals and chelating metals. Our results suggest that phenolic compounds are a major factor correlated with health benefits. However, our toxicity results justify concerns regarding the consumption of high doses that become unsafe and harmful to the human health. Therefore, further animal and human trials are needed to unravel ensure safe dosage of sweet cherry fractions and to incite their use to enrich food and nutraceutical or pharmaceutical products.

Author Contributions: Ana C. Gonçalves performed the research and wrote the paper. M. Rodrigues and Adriana O. Santos contributed new reagents and analytical tools. Gilberto Alves and Luís R. Silva designed the research, analyzed the data, and edited the paper. All authors approved the manuscript in its current form.

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