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Nutritional, chemical and antioxidant/pro-oxidant profiles of silverskin, a coffee roasting by-product

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ABSTRACT

Coffee silverskin (a coffee roasting by-product) contains high amounts of dietary fibre (49% insoluble and 7% soluble) and protein (19%). Potassium (~5 g/100 g), magnesium (2 g/100 g) and calcium (0.6 g/100 g) are the major macrominerals. The vitamin E profile of silverskin comprises α-tocopherol, β-tocopherol, γ-tocopherol, δ-tocopherol, β-tocotrienol, γ-tocotrienol, and δ-tocotrienol. The fatty acid profile is mainly saturated (C16:0 and C22:0), but the total amount of fat is low (2.4%). Caffeine (1.25 g/100 g), chlorogenic acid (246 mg/100 g), and 5-hydroxymethylfurfural (5.68 mg/100 g) are also present in silverskin. Total phenolics and flavonoids are partially responsible for the *in vitro* antioxidant activity. Silverskin extracts protected erythrocytes from oxidative AAPH- and H_2O_2-induced hemolysis, but at high concentrations a pro-oxidant effect on erythrocyte morphology was observed.

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1. Introduction

In recent years (2015/16), the global coffee production reached 145 million of 60 kg bags (ICO, 2016). Along with this production, a great amount of residues (*e.g.* husks, hulls, defective beans, coffee silverskin, and spent coffee grounds) is generated. They constitute a source of contamination and a severe environmental problem, especially due to their richness in phytotoxic and/or *anti*-nutrient compounds (*e.g.* caffeine, tannins, and polyphenols) that can limit their direct use in soil or feed applications. However, they may be a good source of bioactive compounds that can be extracted and further used in food, cosmetics or pharmaceuticals (Mussatto, Machado, Martins, & Teixeira, 2011; Oliveira & Franca, 2015). Thus, the management and re-use of these high added-value residues can contribute to the sustainable development of the coffee chain itself, the global economy and, simultaneously, to a greener environment.

Based on this, it is crucial to make a general effort to valorize the by-products that result from coffee processing. In this context, coffee silverskin (the by-product of coffee roasting) emerges as a particularly interesting product. It is a thin tegument that constitutes the outer layer of raw coffee beans which is detached during the expansion of the beans when subjected to the high temperatures of roasting.

To date, coffee silverskin has been mainly used as direct fuel (*e.g.* firelighters), for composting and soil fertilization but, recently, several innovative approaches have been suggested, essentially based on its richness in dietary fibre, phenolic compounds and other antioxidants, such as melanoidins (Ballesteros, Teixeira, & Mussatto, 2014; Borrelli, Esposito, Napolitano, Ritieni, & Fogliano, 2004; Costa et al., 2014; Mussatto et al., 2011). For instance, Mussatto et al. (2011) suggested the incorporation of coffee silverskin in flakes, breads, biscuits and snacks. Also, Pourfarzad, Mahdavian-Mehr, and Sedaghat (2013) used this by-product to improve quality, shelf life, and sensorial properties of Barbari flat bread, while reducing its caloric density and increasing the dietary fibre content. In turn, Martinez-Saez et al. (2014) used silverskin to prepare a novel antioxidant beverage for body weight control, while Rodrigues, Matias, Ferreira, Amaral, and Oliveira (2016)

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reported the successful use of silverskin as a cosmetic active ingredient with results similar to hyaluronic acid in the improvement of skin hydration and firmness.

Different classical spectrophotometric methods (DPPH·-scavenging ability, ferric-reducing antioxidant power, trolox equivalent antioxidant capacity, among others) have been used to evaluate the antioxidant properties of coffee silverskin (Ballesteros et al., 2014; Borrelli et al., 2004; Costa et al., 2014; Napolitano, Fogliano, Tafuri, & Ritieni, 2007; Narita & Inouye, 2012). In addition, Mesías et al. (2014) found that, in association with its antioxidant activity, silverskin aqueous extracts also have *in vitro* antiglycative properties, protecting against the formation of advanced glycation end-products and trapping of carbonyl-reactive species, such as methylglyoxal.

In this work, we analysed, for the first time, the vitamin E (an antioxidant liposoluble vitamin) profile of coffee silverskin, as well as, the capacity of this by-product to protect human erythrocytes from oxidative damage under oxidative stress conditions. This can suggest the ability of silverskin to protect cells against oxidative injuries in a real biological system. Coffee silverskin was also characterized regarding its nutritional composition, including soluble and insoluble fibre, macromineral content, and fatty acid profile. Caffeine, chlorogenic acid, total phenolics, and total flavonoid contents, as well as *anti*-radical scavenging capacity and ferric-reducing antioxidant power were also assessed.

2. Material and methods

2.1. Reagents and standards

For the macronutrients analysis, all analytical grade reagents were purchased from Panreac (Barcelona, Spain) and Merck (Darmstadt, Germany). The standards, sodium, potassium, calcium, magnesium, iron, chlorogenic acid, caffeine and 5-hydroxymethylfurfural, and the Supelco 37 Component, FAME Mix, were all obtained from Sigma–Aldrich (St. Louis, USA). Tocopherols (α, β, γ and δ) and tocotrienols (α, β, γ and δ) were purchased from Calbiochem (La Jolla, CA, USA) and tocol was obtained from Matreya Inc. (Pennsylvania, USA). HPLC grade solvents were obtained from Sigma–Aldrich (St. Louis, USA) or Merck (Darmstadt, Germany).

For the antioxidant assays, gallic acid, epicatechin, ferrous sulfate heptahydrate and trolox, as well as the Folin-Ciocalteu's reagent, 2,2-diphenyl-1-picrylhydrazyl radical (DPPH·), sodium nitrite, ferric chloride, aluminium chloride, 2,4,6-tripyridyl-s-triazine (TPTZ), 2,2′-azobis(2-amidinopropane dihydrochloride (AAPH), sodium acetate, and sodium azide were all acquired from Sigma-Aldrich (St. Louis, USA). Anhydrous sodium carbonate, sodium hydroxide, absolute ethanol and hydrogen peroxide were purchased from Merck (Darmstadt, Germany). All other reagents were of analytical grade. Ultrapure water was obtained in a Milli-Q water purification system (Millipore, Bedford, MA, USA).

2.2. Sample and sample preparation

Silverskin (∼2.5 kg) representing the major roasting by-product of a national coffee industry, was kindly supplied by BICAFÉ, Torrefação e Comércio de Café, Lda (Portugal). After reception, the sample was stored in a fresh and dry place, protected from light.

2.3. Nutritional analysis

Moisture was determined using an infrared balance (Scaltec model SMO01, Scaltec Instruments, Heiligenstadt, Germany). The remaining nutritional analyses were performed according to AOAC procedures (AOAC, 2012). Briefly, ashes were quantified after incineration at 500 °C. Total lipids and protein were determined by the Soxhlet and the Kjeldahl methods, respectively. Fibre was analysed through enzymatic-gravimetric procedures. Non-fibre carbohydrates were calculated by difference.

2.4. Macromineral composition

The mineral analysis was performed according to Pinto, Almeida, Aguiar, and Ferreira (2014). Briefly, silverskin was digested in an MLS-1200 Mega microwave digestion unit (Milestone, Sorisole, Italy), in the presence of HNO_3 (65%) and H_2O_2 (30%). After digestion, the mixture was diluted to 25 ml with ultrapure water. Macromineral contents were determined using a Perkin Elmer (Überlingen, Germany) 3100 flame (air-acetylene) atomic absorption spectrometer. Calibration standards were prepared from 1000 mg/l single-element standard solutions of Ca, Na, Mg, Fe, and K.

2.5. Fatty acids profile

Silverskin lipids were obtained by Soxhlet extraction during 2.5 h with *n*-hexane, which was subsequently evaporated under a nitrogen stream. The fatty acids were derivatized according to Fernandes et al. (2012) to form the corresponding fatty acid methyl esters (FAMEs). Briefly, the sample was heated at 100 °C for 10 min in the presence of 0.5 M KOH in methanol. Thereafter, 2 ml of boron trifluoride solution (14% in methanol) were added and the mixture was heated at 100 °C for 30 min. After cooling, 2 ml of deionized water and 5 ml of *n*-hexane were also added. The mixture was vortexed and centrifuged. The upper layer was transferred to a 4 ml vial and anhydrous sodium sulfate was used to eliminate any remaining water. This mixture was further vortexed and centrifuged. The supernatant was analysed in a gas chromatograph (GC) Shimadzu GC-2010 Plus (Shimadzu, Tokyo, Japan) coupled with a split/splitless Shimadzu AOC-20i auto-injector (Shimadzu, Tokyo, Japan) and a flame ionization detector (FID) (Shimadzu, Tokyo, Japan). A CP-Sil 88 silica capillary column (50 m × 0.25 mm i.d, 0.20 μm film thickness; Varian, Middelburg, Netherlands) was used to achieve compound separations. The injection was performed in the split mode (1:25), and helium was used as carrier gas. The injection volume was 1.0 μl. The column temperature was programmed as follows: 80 °C, 5 min;, from 80 to 200 °C, at 5 °C/min and then held 5 min and from 200 to 220 °C, at 4 °C/min and held 15 min. Injector and detector temperatures were 250 and 270 °C, respectively. FAMEs were identified by comparison with standards (FAME 37, Supelco). Data were analysed, using the Shimadzu software GC Solution (Shimadzu, Tokyo, Japan). The results were expressed as relative percentage of each fatty acid.

2.6. Chromatographic analyses

2.6.1. HPLC equipment

The chromatographic analyses were conducted in an HPLC integrated system composed of an AS-2057 automated injector, a PU-2089 pump, and a MD-2018 multi-wavelength diode array detector (DAD), and a FP-2020 fluorescence detector (FD), from Jasco, Japan. Data were analysed with JASCO-Chrom NAV Chromatography Software (Jasco, Japan).

2.6.2. Vitamin E profile

Silverskin lipids were obtained by Soxhlet extraction during 2.5 h with *n*-hexane (Pimentel et al., 2014), which was then evaporated under a nitrogen stream. A small amount of lipids (∼50 mg) was mixed with the 20 μl of tocol (1 mg/ml) and *n*-hexane. The

mixture was centrifuged (Heraeus Sepatech Biofuge Pico, Heraeus Instruments, Schwerte Germany) and the supernatant was analysed by HPLC. The chromatographic separation was carried out in a normal phase Supelcosil ™ LC-SI (75 mm × 3 mm, 3 μm) column (Supelco, Bellefonte, PA) with an isocratic eluent system (*n*-hexane/1,4-dioxane (98:2)), at a flow rate of 0.7 ml/min, according to Alves, Casal, and Oliveira (2009). The detection was performed by both DAD and FD (λ_{exc} = 290 nm; λ_{em} = 330 nm). The compounds were identified by their UV spectra and the retention times compared to standards. Quantification was achieved using the internal standard method and the fluorescence signals. The analysis was performed in triplicate.

2.6.3. Caffeine, chologenic acid and 5-hydroxymethylfurfural contents

The chemical compounds were extracted, in triplicate, according to Costa et al. (2014), using a small amount of ground silverskin (1.00 g) and 50 ml of a hydroalcoholic solvent (1:1), under magnetic stirring, for 60 min, at 40 °C. The final extracts were filtered and analysed by HPLC. The compounds were separated, using a reversed-phase Tracer-Excel ODSA (250 mm × 4 mm, 5 μm) from Teknokroma (Barcelona, Spain). A solvent system of 0.5% acetic acid:water (A) and methanol (B) was used as described by Alves et al. (2010). Caffeine was monitored at 274 nm, 5-hydroxymethylfurfural at 280 nm, and chlorogenic acid at 320 nm. The compound identifications were performed by comparing retention times and co-elution with authentic standards, and by UV absorption spectral analysis.

2.7. Antioxidant profile

2.7.1. Extracts preparation

Two types of extracts were prepared, using 100% water and 100% absolute ethanol. In brief, a carefully weighed amount of silverskin (~1 g) was extracted with 50 ml of each solvent, at room temperature, with magnetic stirring, during 60 min. Each extraction was performed in triplicate.

2.7.2. Estimation of antioxidant compounds

2.7.2.1. Total phenolics content. Total phenolics were determined, in triplicate, according to Alves et al. (2010). Briefly, 500 μl of extract were mixed with 2.5 ml of Folin-Ciocalteu reagent (1:10) and 2 ml of sodium carbonate solution (7.5%, m/v). The mixture was then incubated during 15 min at 45 °C, followed by 30 min at room temperature, and always protected from light. Absorbance was then measured at 765 nm. A calibration curve was prepared with gallic acid (10–100 mg/l; *r* = 0.9997) and results were expressed as mg of gallic acid equivalents (GAE)/l of extract.

2.7.2.2. Total flavonoids content. Total flavonoids were determined in triplicate according to Costa et al. (2014). In brief, 1 ml of extract was mixed with 4 ml of distilled water and 300 μl of sodium nitrite (25%). After 5 min of incubation at room temperature, 300 μl of 10% $AlCl_3$ were added to the mixture. After waiting 1 min, 2 ml of sodium hydroxide (1 M) and 2.4 ml of ultrapure water were also added. Absorbance measurements were performed at 510 nm. A calibration curve was prepared with epicatechin (50–450 mg/l; *r* = 0.9998), and results were expressed as mg of epicatechin equivalents (ECE)/l of extract.

2.7.3. Evaluation of antioxidant and pro-oxidant activities

2.7.3.1. DPPH·-scavenging ability. The DPPH·-scavenging ability of silverskin extracts was evaluated according to Costa et al. (2014). The reaction was initiated by transferring 14 μl of a diluted extract (1:10) to 186 μl of a freshly prepared DPPH· solution (9.3×10^{-5} M in ethanol). Absorbance was measured at 525 nm, after 40 min of reaction. The assay was performed in triplicate. A calibration curve was prepared with trolox (25–175 mg/l, *r* = 0.9995) and the DPPH·-scavenging activity was expressed as mg of trolox equivalents/l of extract.

2.7.3.2. Ferric-reducing antioxidant power. The assay was carried out, in triplicate, according to Benzie and Strain (1996), with minor modifications. Briefly, 90 μl of a diluted extract (1:10) were mixed with 270 μl of distilled water and 2.7 ml of FRAP solution (freshly prepared by mixing 0.3 M acetate buffer, 10 mM TPTZ solution, and 20 mM of ferric chloride). The mixture was kept in the dark for 30 min at 37 °C and, afterwards, absorbance was measured at 595 nm. The results were compared with a standard curve prepared with different concentrations of ferrous sulfate (50–450 mg/l, *r* = 0.9998) and reducing antioxidant power was expressed as mg of ferrous sulfate equivalents (FSE)/l extract.

2.7.3.3. Effect on erythrocyte oxidative-induced hemolysis.

2.7.3.3.1. Preparation of erythrocyte suspensions. Blood was obtained from four healthy volunteers, aged 35–45 years, by venipuncture, and collected into tubes containing ethylenediaminetetraacetic acid (EDTA) as anticoagulant. Samples were immediately centrifuged at 2000*g* for 10 min; plasma and buffy coat were carefully removed and discarded. Erythrocytes were washed three times with PBS (125 mM NaCl and 10 mM sodium phosphate buffer, pH 7.4) at 4 °C and re-suspended in PBS to obtain erythrocyte suspensions at 2.6% hematocrit, which were used immediately after preparation.

2.7.3.3.2. Preparation of sample extracts. The extracts prepared in the Section 2.7.1 (aqueous and ethanolic) were lyophilized or evaporated under a nitrogen stream, respectively, and the residue was re-suspended in PBS at different concentrations: 100, 50, 25, 12.5, 6.25, 3.13, 1.56, 0.78, 0.39 e 0.20 g/l.

2.7.3.3.3. Hemolysis inhibition. Hemolysis was induced by using AAPH or H_2O_2 as oxidant agents. All sets of experiments included a negative (erythrocytes in PBS) and a positive (erythrocyte in PBS with AAPH or H_2O_2) control. In order to evaluate hemolysis inhibition, the erythrocyte suspension was incubated with an extract aliquot, always obtaining a final concentration of 60 mM AAPH or 1 mM H_2O_2, and a hematocrit of 2.0%. Sodium azide (final concentration of 1 mM) was added in the H_2O_2 assay to inhibit catalase activity. Incubation was carried out at 37 °C for 2 h, under gentle shaking. By this method, the individual extracts were tested in the presence of each initiator radical (AAPH or H_2O_2).

Hemolysis level was determined spectrophotometrically, according to Ko, Hsiao, and Kuo (1997). After 2 h of incubation, an aliquot of the erythrocyte suspension was diluted with 20 volumes of PBS and centrifuged (1200*g* for 10 min). The absorption (A) of the supernatant was read at 540 nm. The absorption (B), corresponding to a complete hemolysis, was measured after centrifugation of an aliquot of erythrocyte suspension previously treated with 20 volumes of ice-cold distilled water. The percentage of hemolysis was then calculated (A/B × 100). The percentage of hemolysis inhibition was calculated, taking into account the hemolysis of the positive control tube as 0% of inhibition. Each control and sample test was analysed in triplicate.

2.7.3.3.4. Evaluation of oxidative status of hemoglobin and erythrocyte morphology evaluation. Spectral scans (480–650 nm) were performed to evaluate the oxidative status of hemoglobin in the hemolysates. Given the dark coloration of silverskin extracts, it was not possible to perform the spectrophotometric readings when higher concentrations of silverskin PBS-reconstituted extracts were used (50 and 100 g/l). Thus, erythrocyte morphologic changes associated with hemolysis were evaluated. For that, aliquots (50 μl) of incubated erythrocyte suspensions with AAPH or H_2O_2 (in the presence or absence of silverskin extracts) were

diluted (1:50) and disposed on a slide with a coverslip, for optical microscopy evaluation.

2.8. Statistical analysis

All experiments were performed in triplicate and data were expressed as means ± standard deviation. Data were analysed, using the programme SPSS 21.0 for Windows (SPSS, Inc., Chicago, IL). One-way ANOVA was used to assess significant differences between samples, followed by Tukey's HSD or Dunett T3 *post hoc* tests (based on the equality of the variances) to make pairwise comparisons between means. The level of significance for all hypothesis tests (p) was 0.05.

3. Results and discussion

The nutritional profile of the coffee silverskin analysed in this study is described in Table 1. This by-product is composed of a high amount of total fibre (56%), of which 87% is insoluble and only 13% is soluble. Coffee silverskin is also very rich in protein (19%), and minerals (8% of ash). Mineral composition of ashes, consisted mainly of potassium (~5 g/100 g silverskin), magnesium (2 g/100 g) and calcium (0.5 g/100 g) (Table 1). Our results are in accordance with those published by Borrelli et al. (2004) who also found 19% of protein and 62% of dietary fibre in silverskin samples. The macromineral profile found in our study differs slightly from that described by Ballesteros et al. (2014), who reported 5% of ash, containing mainly potassium (2.1 g/100 g of dry silverskin), calcium (0.9 g/100 g), and magnesium (0.3 g/100 g), differences that are mainly due to the coffee species and origins of the provided silverskin.

Table 1
Chemical composition of coffee silverskin.

Coffee silverskin composition	
Moisture (g/100 g)	4.76 ± 0.10
Ash (g/100 g)	8.34 ± 0.04
Fat (g/100 g)	2.42 ± 0.10
Protein (g/100 g)	18.8 ± 0.30
Dietary fibre (g/100 g)	56.4 ± 0.70
Insoluble fibre (g/100 g)	49.1 ± 0.44
Soluble fibre (g/100 g)	7.30 ± 0.07
Carbohydrates (g/100 g)	5.80 ± 0.20
Macrominerals (mg/100 g)	
K	4977 ± 151
Mg	2002 ± 72
Ca	584 ± 62
Fe	41.8 ± 2.69
Na	5.32 ± 0.14
Vitamin E (mg/100 g)	
α-tocopherol	2.25 ± 0.14
β-tocopherol	0.59 ± 0.05
γ-tocopherol	0.09 ± 0.01
δ-tocopherol	0.03 ± 0.01
α-tocotrienol	n.d.
β-tocotrienol	0.95 ± 0.07
γ-tocotrienol	0.17 ± 0.01
δ-tocotrienol	0.09 ± 0.01
Caffeine (g/100 g)	1.25 ± 0.03
Chlorogenic acid (mg/100 g)	246 ± 0.2
5-Hydroxymethylfurfural (mg/100 g)	5.68 ± 0.42

Data are expressed per 100 g of fresh weight; n.d., not detected.

Table 2
Fatty acids profile of coffee silverskin.

Fatty acids (relative %)	Coffee silverskin
C12:0	0.08 ± 0.01
C14:0	1.88 ± 0.06
C15:0	0.43 ± 0.01
C16:0	21.9 ± 0.20
C16:1	0.25 ± 0.01
C17:0	0.35 ± 0.01
C18:0	5.93 ± 0.01
C18:1*n*9c	6.85 ± 0.07
C18:2*n*6c	24.4 ± 0.11
C20:0	13.5 ± 0.11
C18:3*n*3	3.68 ± 0.02
C21:0	0.66 ± 0.01
C22:0	15.4 ± 0.20
C23:0	0.66 ± 0.01
C24:0	4.04 ± 0.06
SFA	64.8 ± 0.16
MUFA	7.10 ± 0.07
PUFA	28.1 ± 0.13

The lipid of the silverskin analysed in this study consisted of about 2.4 g/100 g, in complete accordance with the values described by Napolitano et al. (2007) that ranged from 1.6% to 3.3%. The lipid, in a previous study by Toschi, Cardenia, Bonaga, Mandrioli, and Rodriguez-Estrada (2014) had triacylglycerols as the major components (48%), followed by free fatty acids (21%), esterified sterols (15%), free sterols (13%), and diacylglycerols (4%).

Table 2 shows the fatty acid profile of coffee silverskin obtained in this study. Summarizing, C18:2*n*6c was the main fatty acid found (24%), followed by C16:0 (22%), C22:0 (15%), and C20:0 (14%). Although the relative percentages varied slightly from those described by Toschi et al. (2014) (29, 28, 11, and 11%, respectively), both profiles are quite similar, especially regarding these main compounds. Nevertheless, in relation to minor fatty acids, we also found C12:0, C21:0, C23:0, while C20:4 was not detected (Table 2), in contrast to data published by Toschi et al. (2014). In general, coffee silverskin presents mainly saturated fatty acids (65%), followed by polyunsaturated (28%) and monounsaturated (7%) ones (Table 2).

Vitamin E content of coffee silverskin, as far as we know, has not previously been described. Vitamin E is a lipossoluble vitamin composed of eight naturally occurring and structurally related vitamers, that contain a chromanol ring with dissimilar substitution patterns of methyl groups (α-, β-, γ-, and δ-), and a saturated or unsaturated 16-carbon phytyl side chain (in tocopherols and tocotrienols, respectively) (Schneider, 2005). α-Tocopherol is the most biologically active form, with 100% of vitamin E activity, and the biological effects are mostly due to antioxidant properties (Eitenmiller & Landen, 1999). Table 1 shows the Vitamin E profile and the content of each vitamer found in this by-product. The main compound was α-tocopherol (2.25 mg/100 g), followed by β-tocotrienol (0.95 mg/100 g), δ-tocopherol being the minor vitamer. α-Tocotrienol was not detected. Comparatively to coffee beans, which contain only α- and β-tocopherol and mean total vitamin E amounts ranging between ~3 and 11 mg/100 g, for arabica and robusta coffees, respectively) (Alves et al., 2009), the silverskin analysed in this study presented a more complete profile with seven different vitamers composing its vitamin E profile (total vitamin E content: 4.17 mg/100 g) (Table 1).

Other important antioxidant compounds present in coffee silverskin (which also exist in coffee beans) are chlorogenic acid (5-*O*-caffeoylquinic acid) (Alves et al., 2010; Costa et al., 2014) and caffeine. Indeed, besides its widely known stimulating effects, caffeine also presents the ability to inhibit lipid peroxidation induced by reactive oxygen species, namely hydroxyl radical ($^{\cdot}OH$), peroxyl radical ($ROO^{\cdot}$), and singlet oxygen ($^1O_2$) (Devasagayam, Kamat, Mohan, & Kesavan, 1996). The coffee silverskin analysed in this study contained 246 mg of chlorogenic acid and 1.25 g of caffeine per 100 g of sample, slightly higher values than those obtained by Narita and Inouye (2012): 110 mg of 5-O-caffeoylquinic acid

Table 3
Total phenolics and flavonoid contents and *in vitro* antioxidant activity (DPPH. inhibition and FRAP assays) of aqueous and ethanolic silverskin extracts.

Type of extract	Bioactive compounds		Antioxidant activity	
	Total phenolics (mg GAE/l)	Total flavonoids (mg ECE/l)	DPPH. inhibition (mg TE/l)	FRAP (mg FSE/l)
Aqueous	173 ± 2.9[a]	86.3 ± 2.0[b]	386 ± 13[b]	1074 ± 164[a]
Ethanolic	85.1 ± 2.1[b]	103 ± 3.8[a]	519 ± 37[a]	1031 ± 92.9[a]

Results are presented as means ± standard deviation of triplicate extractions. Different letters in each column represent significant differences at $p < 0.05$. GAE, gallic acid equivalents; ECE, epicatechin equivalents; TE, trolox equivalents; FSE, ferrous sulfate equivalents.

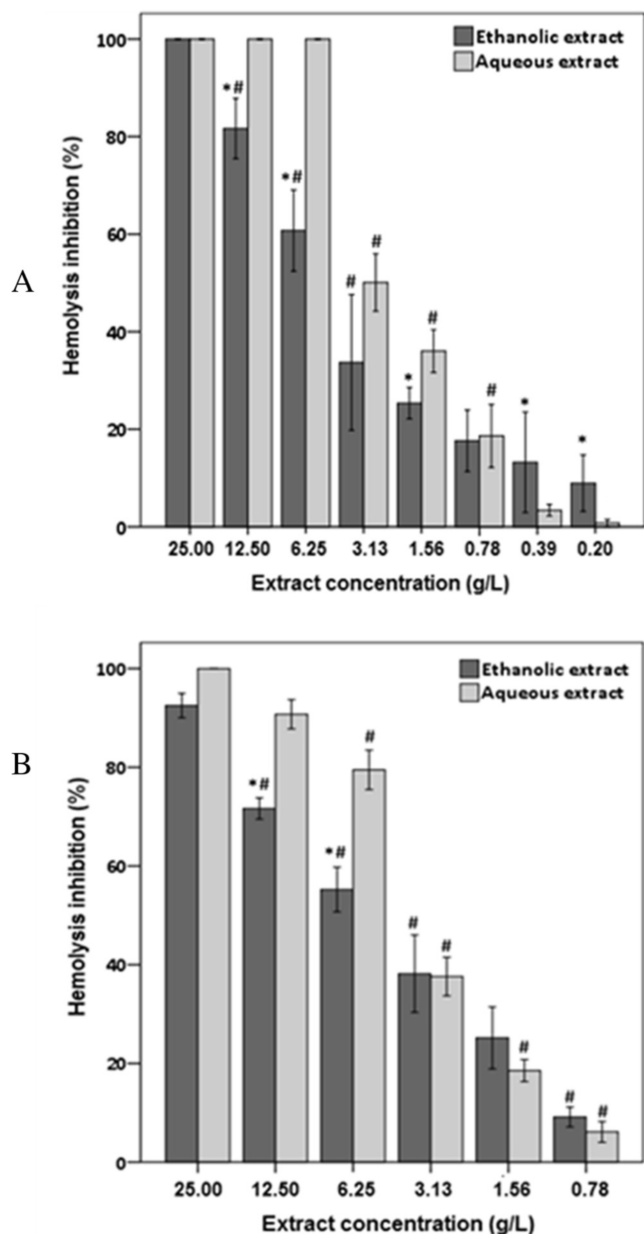

Fig. 1. Effect of different concentrations of silverskin extracts (ethanolic and aqueous) on the inhibition of erythrocyte hemolysis (means ± SEM) at 2% of hematocrit, under AAPH induced oxidative stress (A) or H_2O_2-induced oxidative stress (B). Legend: $^{\#}p < 0.05$ vs previous concentration; $^{*}p < 0.05$ vs aqueous extract.

and 0.44 g of caffeine per 100 g of silverskin. For caffeine, Toschi et al. (2014) reported 0.77–1.03 g/100 g, while Bresciani, Calani, Bruni, Brighenti, and Del-Rio (2014) reported 0.89–1.1 g/100 g, values closer to our own. In the case of caffeine, these differences can be especially due to the coffee species that provided silverskin. However, in the case of chlorogenic acid, the roast degree achieved during bean roasting has also to be considered, since 5-*O*-caffeoylquinic acid is thermally degraded to the respective lactone (Alves et al., 2010).

Hydroxymethylfurfural is formed during roasting, and is known as a potentially harmful compound (Janzowski, Glaab, Samimi, Schlatter, & Eisenbrand, 2000). Alves et al. (2010) reported levels of 0.68–2.60 mg/espresso coffee (30 ml), which corresponds to 10–40 mg/100 g of roasted coffee beans, considering that extraction to the brew was complete. Curiously, the higher levels were found in lighter roasted arabica coffees, suggesting that hydroxymethylfurfural content is influenced by coffee species and is formed at the beginning of the roast, being then degraded to other chemical compounds during the Maillard reactions. In this work, we found ~6 mg of hydroxymethylfurfural per 100 g of silverskin, a value that is below those reported by Alves et al. (2010) for coffee, and also below that described by Narita and Inouye (2012) for silverskin (200 mg/100 g). The significant difference between our value and that reported by Narita and Inouye (2012) could eventually be due, as mentioned above, to differences in the coffee species and roasting, but also in the extraction method, since those authors used subcritical water for extraction.

The total phenolic and flavonoid contents of silverskin extracts, as well as the antioxidant activity, using both the DPPH· inhibition and FRAP assays, were all evaluated and the results are presented in Table 3. Total phenolics averages were 85.1 mg GAE/l of extract (4.3 mg GAE/g silverskin) and 173 mg GAE/l of extract (8.7 mg GAE/g silverskin) for ethanolic and aqueous extracts, respectively. Similar phenolic contents (6–7 mg GAE/g silverskin) were reported by other authors for aqueous extracts (1 g/50 ml) prepared at 25 and 80 °C. In addition, it was shown that subcritical water can increase the extraction of silverskin antioxidants (Narita & Inouye, 2012). However, this requires expensive and specialized equipment, not always available in analytical laboratories. Ballesteros et al. (2014), using ethanol and different solvent (ml) to solid (g) ratios (10, 25, and 40), obtained phenolic concentrations ranging from ~5.5 to ~13.0 mg GAE/g of silverskin. For flavonoid contents, ethanol was the best extraction solvent (103 mg ECE/l or 5.1 mg ECE/g), clearly due to the solubility characteristics of this group of compounds. In the aqueous extracts, 86.3 mg ECE/l (4.3 mg ECE/g) were found.

Several studies support the hypothesis that antioxidants might have a beneficial role in the prevention of chronic diseases associated with oxidative stress (Lushchak, 2011; Paiva-Martins et al., 2009; Pandey & Rizvi, 2009; Scalbert, Johnson, & Salmarsh, 2005). Currently, there are several methods for the evaluation of total antioxidant activity in foodstuffs, each one with its advantages and limitations. Because of that, it is advisable to use more than one method to study the antioxidant profile. In this work, two chemical-based methods (namely, DPPH·-scavenging ability and ferric-reducing antioxidant power (FRAP)), together with an *in vitro* cellular assay using erythrocytes, were performed in order to assess different and complementary mechanisms of antioxidant activity.

The DPPH·-scavenging assay is used as a basic screening method to study the antiradical activity of a large variety of bioactive natural compounds. The radical can be neutralized, either by direct reduction (via electron transfer) or by radical quenching (via H atom transfer). In turn, in the FRAP assay, the reduction of ferric 2,4,6-tripyridyl-s-triazine (TPTZ) to a coloured product occurs. This reaction detects several compounds with redox potentials lower than 0.7 V (the redox potential of Fe^{3+}-TPTZ), being a reasonable screen for the ability to maintain the redox status in cells or tissues (Alves et al., 2010; Benzie & Strain, 1996; Costa et al., 2014). However, this method cannot detect compounds that act by radical quenching (H transfer) since only the electron transfer mechanism occurs. As expected, owing to its antioxidant composition, both models showed the antioxidant efficiency of silverskin extracts. Nevertheless, the extracts behaved differently, with the type of solvent significantly affecting the general antioxidant capacity (Table 3). While no significant differences were found between FRAP values obtained for the two types of silverskin extracts, in the DPPH· inhibition assay, ethanolic extracts showed higher values. This shows that silverskin contains antioxidant compounds with different mechanisms of action, and suggests that ethanolic extracts are richer in compounds that act by radical quenching (via H atom transfer). In addition, it was possible to correlate flavonoids content with DPPH· inhibition, but total phenolics content showed no correlation in both assays, suggesting that compounds other than phenolics are also responsible for the antioxidant activity of silverskin. In fact, as already described for coffee beans, silverskin (being a roasting by-product) also contains Maillard reaction products, such as melanoidins (Borrelli et al., 2004), which have also been described as relevant antioxidant compounds.

In this work, the additional use of erythrocytes to evaluate the antioxidant activity of silverskin shows major advantages. Anucleated, and with no cytoplasmatic organelles, these cells present poor mechanisms of repair and biosynthesis, suffering and accumulating oxidative lesions whenever oxidative stress occurs. Nevertheless, they are equipped with several antioxidants (*e.g.* glutathione, α-tocopherol and ascorbate). If reactive oxygen species are overproduced, or if the endogenous antioxidant defences are impaired, erythrocytes will be subjected to oxidative stress, which can lead to damage of membrane and hemoglobin, and, consequently, to hemolysis (Paiva-Martins et al., 2009; Van der Berg, Op den Kamp, Lubin, Roelofsen, & Kuypers, 1992). When the capacity of protective hemoglobin-scavenging mechanisms is saturated, the levels of cell-free hemoglobin increase in the plasma (Rother, Bell, Hillmen, & Gladwin, 2005). Whenever the hemoglobin is released from erythrocytes, it is potentially harmful because it can be converted into oxidized forms, powerful promoters of oxidative processes in blood (Bamm, Tsemakhovich, & Shaklai, 2003; Rother et al., 2005). The ability of silverskin extracts to inhibit induced oxidative hemolysis of human erythrocytes caused by two different initiator agents (radical 2,2′-azobis (2-amidinopropane) dihydrochloride (AAPH) and hydrogen peroxide (H_2O_2)) was studied and results are presented in Fig. 1. This assay is biologically more relevant than the first two chemical-based assays, since a cell model is used as a target for oxidative damage. Indeed, erythrocytes, as oxygen carriers, and cells with high concentrations of polyunsaturated fatty acids in their membranes, may be considered a major target for free radical attack (Ajila & Rao, 2008). Fig. 1A and B show, respectively, the antioxidant effect of silverskin extracts on human erythrocytes exposed to the initiators AAPH and H_2O_2. The extracts protected erythrocytes in a significant way and in a dose-dependent manner. Indeed, a significant protective effect of silverskin extracts was found at the concentration of 25 g/l, in both methods (AAPH and H_2O_2-induced oxidative stress) and with both types of extracts (ethanolic and aqueous). This protective effect decreased significantly with the decrease of the extract concentration, being undetectable at concentrations lower than 0.20 g/l for AAPH and 0.78 g/l for H_2O_2-induced oxidative stress. The difference in the protective effect against these two initiators could be related to the fact that H_2O_2, a physiological oxidizing agent, attacks not only the outside of cells but also inside them. H_2O_2 can easily cross the cell membrane, requiring high extract concentrations to achieve the same level of hemolysis inhibition as obtained with AAPH. When comparing the two types of extracts, there is a significantly lower protective effect of ethanolic extracts, eventually related to their lower

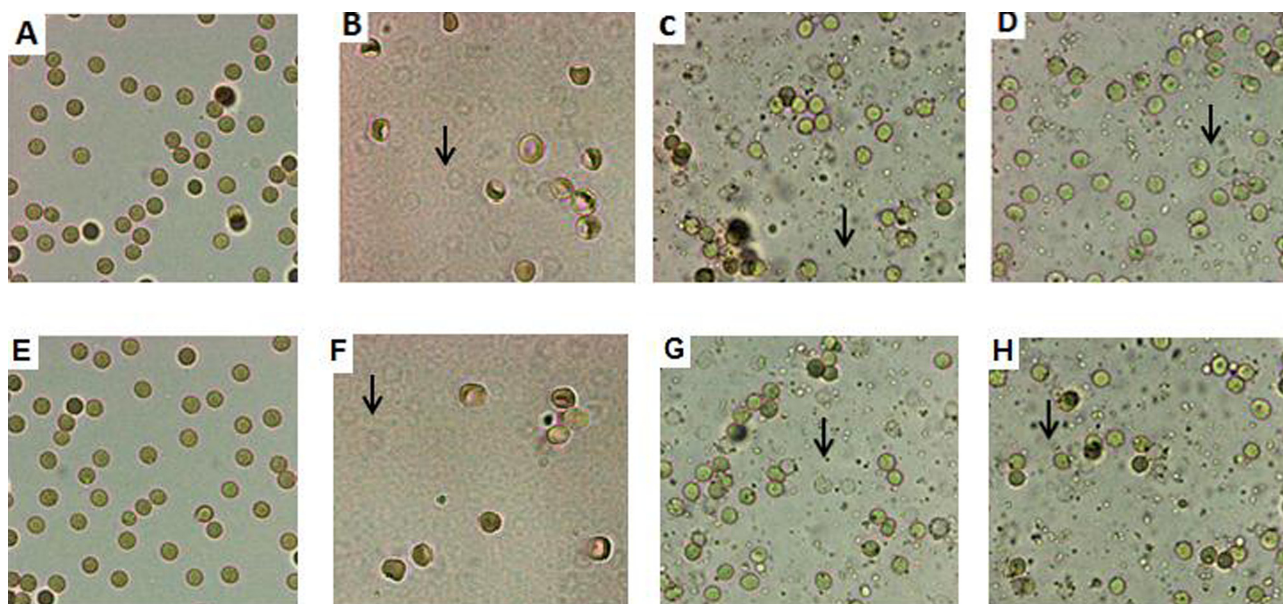

Fig. 2. Optical microscopic evaluation of erythrocyte morphology. A) Control just with erythrocytes; B) Positive control with erythrocytes and AAPH, with low cell density and free hemoglobin erythrocytes (ghost cells); C) Erythrocytes incubated with 100 g/l of silverskin extract and AAPH, with ghost cells; D) Erythrocytes incubated with 50 g/l of silverskin extract and AAPH, with ghost cells; E) Control just with erythrocytes; F) Positive control with erythrocytes and H_2O_2, with low cell density and free hemoglobin erythrocytes (ghost cells); G) Erythrocytes incubated with 100 g/l of silverskin extract and H_2O_2, with ghost cells; H) Erythrocytes incubated with 50 g/l of silverskin extract and H_2O_2, with ghost cells.

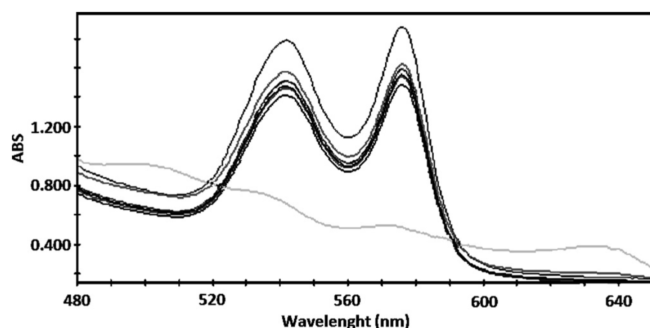

Fig. 3. Spectral scans (480–650 nm) of lysed erythrocyte suspensions after 2 h of incubation (black lines) in the presence of AAPH and different concentrations of silverskin extracts (25.0, 12.5, 6.25, 3.13, 1.56 and 0.78 g/l), and control assay (erythrocytes without AAPH and silverskin extract). The oxy-hemoglobin peaks disappeared in the presence of AAPH without silverskin extract (gray line).

total phenolic content (Table 3). The effect of high concentrations of silverskin extracts (50 and 100 g/l) was evaluated by optical microscopy, since the dark coloration obtained made the spectrophotometric reading impossible. Thus, after 2 h of incubation the erythrocyte morphology was evaluated, showing the presence of ghost cells (free hemoglobin erythrocytes) (Fig. 2). These results suggest that for the highest extract concentrations (50 and 100 g/l) the antioxidant capacity disappears, creating, instead, a pro-oxidant effect. Below these concentrations, the silverskin phytochemicals seem to inhibit the lipid peroxidation by trapping the radicals before they attack the lipid molecules of the erythrocyte membrane. This breaks the free radical chain reaction and inhibits subsequent oxidative hemolysis. Spectral scans, determined between 480 and 650 nm, were also performed for extract concentrations below 25.0 g/l to evaluate the oxidation status of erythrocyte hemoglobin. The results showed no changes in the oxy-hemoglobin peaks (Fig. 3) when compared to the control assay (erythrocytes without silverskin extract and initiator agent), which demonstrated that, for those concentrations, the silverskin extracts have no oxidant effects in hemoglobin. Instead, silverskin extracts interact with erythrocytes, protecting them from oxidative hemolysis initiated by AAPH and H_2O_2. The exact mechanism associated with this protective effect is still unknown; however, we can assume that the ability to protect erythrocytes from hemolysis is related, not only to a radical-scavenging activity, but also to its ability to interact directly with cell membranes (in the outer or inner membrane surface).

4. Conclusions

Based on its chemical composition, namely a very high content in fibre, and especially an insoluble one, protein, macrominerals, and antioxidants, silverskin is a product with a potential for food applications. Although the fatty acid profile is mainly saturated, the total amount of fat is low. In addition, the vitamin E content of silverskin was quantified for the first time, showing a very complete profile composed of seven different vitamers (only α-tocotrienol was not present). Total phenolics and total flavonoids were partially responsible for the antioxidant activity assessed by DPPH· inhibition and ferric-reducing antioxidant power assays, suggesting the influence of other compounds besides phenolics and flavonoids in the antioxidant activity of silverskin (for instance, melanoidins). Moreover, according to our results, silverskin extracts (0.20–25 g/l) were able to inhibit induced hemolysis. However, in excess (50 and 100 g/l), the antioxidant capacity disappeared and a pro-oxidant effect was observed. These results show that, within a certain range of concentration, silverskin extracts can play an important role in cell protection against oxidative injuries. Based on this, further assays should be undertaken to better understand the potential biological activities of this by-product.

Conflicts of interest

The authors state that there are no conflicts of interest.

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