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Castanea sativa Shells: Is Cosmetic Industry a Prominent Opportunity to Valorise This Agro-Waste? †

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Abstract: The purpose of this study was to recover antioxidants from chestnut shells (CS) by Supercritical Fluids Extraction (SFE), aiming their reuse as new cosmetic principles. The antioxidant/antiradical properties and inhibitory activities on hyaluronidase and elastase reinforced the potential use of CS extract as an effective source of anti-aging ingredients. The *in-vitro* assays proved the safeness on HaCaT and HFF-1 cells up to 100 µg/mL. Regarding *ex-vivo* permeation studies, two polyphenols, namely ellagic acid and epicatechin permeated the skin after 8 h of testing. This study emphasizes the skin health effects and safety of CS extract as an anti-aging cosmetic ingredient.

Keywords: chestnut shells; supercritical fluids extraction; anti-aging; cosmetics; skin permeation; *in-vitro* assays

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1. Introduction

Sustainability arises as a pivotal concept in different industries. The demand of new cosmetic active principles recovered from food by-products has clearly boosted due to environmental impacts and bioeconomy concept, aiming to create added value for food wastes. In this way, different research groups have focused on the valorization of by-products produced by natural fruits with huge impact in the Portuguese economy.

Castanea sativa, commonly known as sweet chestnut, produces valuable fruits with unique sensorial, nutritional and pro-healthy properties [1]. Chestnut industry is one of the primary motors of the Portuguese fruit economy [1]. Large amounts of by-products are generated during chestnut processing. Shells are the most abundant and underexploited by-product generated during chestnut peeling. The wealth of chestnut shells (CS) in bioactive compounds, including vitamin E, amino acids and phenolic compounds (phenolic acids, flavonoids and tannins), endowed with interesting biological activities, namely antioxidant, anti-inflammatory, anticancer and antimicrobial properties, reinforced the potential of shells' valorization as a substrate of natural antioxidants, empha

sizing the possible benefits of designing high-added value products with attractive applications in cosmetic field [2–5].

The richness of CS extracts, obtained by sustainable and ecofriendly techniques, in bioactive compounds with health-promoting effects has been demonstrated in previous researches [4,6–8]. The implementation of eco-innovative technologies, such as supercritical fluids extraction (SFE), to recover bioactives has emerged as a priority for the industrial valorization of agro-residues. This study aimed for the first time to (1) screen the antioxidant and antiradical properties of CS extract prepared by SFE-CO_2; (2) study the inhibitory activity on elastase and hyaluronidase; (3) assess the *in-vitro* safety for cosmetic purposes; and (4) characterize the *ex-vivo* permeation profile of bioactive compounds through Franz cells, using human skin as barrier. To the best of our knowledge, this study provides for the first time a comprehensive evaluation of the cosmetic potential of a CS green extract as antioxidant and anti-aging ingredient.

2. Materials and Methods

2.1. Castanea sativa shells

CS were kindly supplied in October 2018 by Sortegel, situated in Bragança, Portugal. Shells were dehydrated at 40 °C for 24 h. The outer shells were separated from the inner shells, ground to a particle size of 1 mm and stored at room temperature in the dark.

2.2. Preparation of Extract by Supercritical Fluids Extraction with CO_2 (SFE-CO_2)

SFE-CO_2 extract was obtained as described by Pinto et al. [4], using a supercritical fluid extractor (Waters Prep SFE-100 system, Milford, CT, USA). A sandwich structure composed of 3 layers was placed into the extraction vessel (100 mL): 5 g of Ottawa sand (first layer), a blend of powdered shells (15 g) and Ottawa sand (30 g) (second layer), and more 5 g of Ottawa sand (third layer). CO_2 (Air Product and Chemicals, Allentown, PA, USA) and ethanol (VWR chemicals, Radnor, PA, USA) were used as supercritical fluid and co-solvent, respectively. The extraction was performed at 60 °C, 350 bar and 15% of co-solvent for 90 min. Then, the extract was evaporated and stored at −20 °C in the dark. The yield of SFE extraction was 1.01% (*w*/*w*) as described in our previous paper [4].

2.3. Antioxidant and Antiradical Properties

The antioxidant and antiradical activities of CS extract were evaluated by three different assays, namely FRAP (IC_{50}, μg/mL), DPPH (% Inhibition) and ABTS (mg ascorbic acid equivalents (AAE)/g on dry weight (dw)) assays. The antiradical activity was also appraised by reactive oxygen and nitrogen species (ROS and RNS, respectively) scavenging assays, namely superoxide anion radical ($O_2^{\bullet -}$), hypochlorous acid (HOCl) and nitric oxide radical ($NO^{\bullet}$).

2.4. Elastase and Hyaluronidase Inhibition Assays

The inhibitory activity of CS extract on hyaluronidase and elastase was determined following the procedures validated by Nema et al. [9]. Regarding the hyaluronidase assay, the medium composed of hyaluronidase (1.50 U/100 mL), 20 mM sodium phosphate buffer (pH 7) with 77 mM sodium chloride and 0.01% bovine serum albumin (BSA) was pre-incubated with extract (5 mL) at 37 °C for 10 min. The assay started by adding hyaluronic acid. After 45 min at 37 °C, the undigested hyaluronic acid was precipitated by acidic albumin solution and the samples were kept at room temperature for 10 min. The absorbance was read at 600 nm. For elastase assay, the *p*-nitroaniline hydrolyzed by elastase from the substrate MeOSuc-Ala-Ala-Pro-Val-pNa was quantified by reading absorbance at 405 nm. MeOSuc-Ala-Ala-Pro-Val-pNa was dissolved in buffer (pH 8) and added to the samples. The solutions were pre-incubated at 37 °C during 10 min before adding elastase. After 30 min at 37 °C, the absorbance was read at 405 nm.

2.5. Skin Cells Viability by In-Vitro Assays

Following the methodology validated by Lameirão et al. [7], the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was employed to assess the effects of CS extract on skin cell lines, namely keratinocytes (HaCaT) from passages 21–23 and fibroblasts (HFF-1) from passages 83–85. Cells were incubated with fresh medium in a 96-well microplate (25×10^3 cells/mL) at 37 °C for 24 h, in 5% CO_2 environment, in the absence and presence of extracts at different concentrations (0.1, 1, 10, 100, and 1000 μg/mL). A Dulbecco's modified Eagle's medium (DMEM) with GlutaMAX™ and supplemented with 0.25 μg/mL amphotericin B, 1% (*v*/*v*) antibiotic–antimitotic mixture (containing 100 U/mL penicillin and 100 μg/mL streptomycin), 1% (*v*/*v*) *L*-glutamine, 10% (*v*/*v*) inactivated FBS and 1% (*v*/*v*) non-essential amino acids was used as culture medium. Then, cells were washed with phosphate buffered saline solution (PBS, pH 7.4) and incubated with MTT reagent for 3 h (37 °C, 5% CO_2). Absorbance was read at 490 nm with background subtraction at 630 nm. Results were presented as percentages of cell viability.

2.6. Skin Permeation of Phenolic Compounds by Ex-Vivo Assay

The permeation of phenolic compounds from CS extract into the skin was studied using Franz diffusion cells (9 mm clear jacketed Franz cell with flat ground joint, 5 mL receptor volume and permeation area of 0.785 cm^2; PermeGear, Inc., USA) and following the procedure of Rodrigues et al. [10]. Considering the previous results, the extract concentration of 1000 μg/mL was selected for this experiment. Human skin with 0.8 mm thickness was used as barrier, obtained from an abdominal surgery of one healthy woman (Department of Plastic Surgery, São João Hospital, Porto, Portugal). The experimental protocol (protocol code: 90_17) was approved by the Bioethics Committee of the São João Hospital and a written informed consent form was provided to the volunteer. The skin biopsy was washed with ultrapure water, the hair and subcutaneous fatty tissue were removed and the skin was kept at −20 °C. The pre-treated skin was placed in the Franz diffusion cell. The receptor compartment was filled with 5 mL of phosphate buffer (0.2 M, pH 7.4) and maintained at 37 °C with continuous stirring (200 rpm). Then, 500 μL of extract were added to the donor chamber. Aliquots of the receptor medium (300 μL) were withdrawn every 30 min for the first 4 h and then every hour until completing 8 h of testing. The same volume (300 μL) of phosphate buffer was added to the receptor compartment to maintain the final volume. The chromatographic analysis of the phenolic profile was done by LC/ESI-MS, according to Fernandes et al. [11]. Results were expressed as the amount of each phenolic compound (μg/g dw) that permeated through the skin.

2.7. Statistical Analysis

Data were presented as mean ± standard deviation of at least three independent experiments. Design Expert version 11 (Stat-Ease Inc., Minneapolis, MN, USA) was employed as mathematical tool for RSM aiming to analyze the responses surface and contour plots, determine the regression equations, and perform the statistical analysis of the experimental design. IBM SPSS Statistics 24.0 software (SPSS Inc., Chicago, IL, USA) was also used to investigate statistical differences among results. One-way ANOVA was applied to determine the differences between samples and post hoc comparisons of the means were carried out using Tukey's HSD test. A denoting significance was accepted for $p < 0.05$. A correlation study was also performed, being selected the Pearson's correlation coefficient 'R' with *p*-value.

3. Results and Discussion

3.1. In-Vitro Antioxidant and Antiradical Activities of CS Extract

Besides the well-described effects of temperature and pressure in SFE efficiency, the co-solvent (ethanol) may also exert a huge influence in the antioxidant activity and

amount of bioactive compounds extracted [4]. CO_2 is effective in the extraction of nonpolar compounds, while a combination with high ethanol (co-solvent) content modifies the selectivity of this technology and, consequently, allows the recovery of polar compounds (e.g., polyphenols) [4]. Table 1 summarizes the results of antioxidant assays.

Table 1. Antioxidant/antiradical activity and radicals scavenging capacity of CS extract prepared by SFE. Values are presented as mean ± standard deviation (n = 3).

	FRAP (IC_{50}, μg/mL)	DPPH (% Inhibition)	ABTS (mg AAE/g dw) [1]	$O_2^{\bullet-}$ (IC_{50}, μg/mL)	HOCl (IC_{50}, μg/mL)	NO• (IC_{50}, μg/mL)
CS extract	204.79 ± 4.33	53.04 ± 6.75	124.84 ± 4.53	49.42 ± 0.41 [2,a]	1.57 ± 0.10 [a]	0.76 ± 0.11 [b]
Catechin	-	-	-	48.99 ± 0.75 [b]	0.18 ± 0.01 [c]	0.95 ± 0.04 [a]
Gallic acid	-	-	-	5.18 ± 0.19 [c]	1.25 ± 0.05 [b]	0.20 ± 0.03 [c]

[1] AAE, ascorbic acid equivalents. [2] result expressed as % inhibition at 500 μg/mL. Different letters in the same column indicate significant differences ($p < 0.05$).

The results evidenced the strong antioxidant and antiradical properties of CS extract with values ranging 124.84 mg AAE/g dw, 53.04% inhibition, and IC_{50} of 204.79 μg/mL for ABTS, DPPH and FRAP assays, respectively. The results of DPPH and FRAP assays were similar or even better than the ones reported by Rodrigues et al. [3] and Squillaci et al. [5] for CS conventional extracts. Additionally, the result for ABTS assay was higher than the ones reported by Zhang et al. for *Citrus reticulata* peels extracts also prepared by SFE (64.24–97.31 mg AAE/g dw) [12]. Among the ROS and RNS screened, the best scavenging efficiencies of CS extract were accomplished for NO• (IC_{50} = 0.76 μg/mL) and HOCl (IC_{50} = 1.57 μg/mL), exhibiting similar results to gallic acid (HOCl: IC_{50} = 1.25 μg/mL; NO•: IC_{50} = 0.20 μg/mL) and catechin (HOCl: IC_{50} = 0.18 μg/mL; NO•: IC_{50} = 0.95 μg/mL) which were tested as positive controls. Significant differences ($p < 0.05$) were observed between extract, gallic acid and catechin for all ROS and RNS assays. Moreover, the HOCl and NO• counteracting power of CS extract was substantially higher than *C. sativa* leaves extract (HOCl: IC_{50} = 63.8 μg/mL; NO•: IC_{50} = 3.10 μg/mL) [13]. A good $O_2^{\bullet-}$ quenching ability was also determined for CS extract (49.42% inhibition). However, *C. sativa* leaves extract achieved a higher scavenging potential for this species (IC_{50} = 13.6 μg/mL) as reported by Almeida et al. [13].

3.2. Hyaluronidase and Elastase Inhibitory Activity

Skin aging is a consequence of intrinsic (such as oxidative metabolism) and extrinsic (mostly UV radiation) factors, leading to the activation of different enzymes, including elastase and hyaluronidase, recognized by their deleterious effects on skin [9]. The inhibition of elastase by CS extract was 33.56% (0.4 mg/mL), while catechin and gallic acid, tested as positive controls, achieved lower results with 17.80% and 15.63% of inhibition, respectively. For the hyaluronidase assay, the CS extract presented an IC_{50} of 54.36 mg/mL. These results were better than the ones reported for olive leaves extracts (hyaluronidase: IC_{50} = 55–100 μg/mL; elastase: ≈ 20% inhibition) [14]. These outcomes suggested that CS extract prepared by SFE may be a potential source of anti-aging ingredients.

Different studies have reported the relationship between phenolic composition and bioactivity of plant-derived extracts, particularly with antioxidant properties and inhibitory activities on elastase and hyaluronidase [9,14,15]. Therefore, antioxidant, anti-elastase and anti-hyaluronidase effects of CS extract obtained by an environmentally friendly technology may be attributed to its phytochemical composition, mainly to ellagic acid, caffeic acid derivative, catechin, epicatechin and epigallocatechin previously identified in CS-SFE extract in our previous study [4].

3.3. Effects towards Skin Cell Lines

The major skin cells are keratinocytes and fibroblasts. In this sense, the screening of the extracts' safeness on these skin cell lines is a primary step in the assessment of novel cosmetic ingredients [15]. The highest concentration tested of CS extract (1000 μg/mL) induced a significant decrease ($p < 0.05$) on HaCaT (15%) and HFF-1 (35%) viability, leading to considerable adverse effects. Overall, the CS extract can be considered as safe for skin application in a concentration range of 0.1–100 μg/mL with viabilities above 60% for both cells and without significant differences ($p > 0.05$).

3.4. Ex-Vivo Permeation of Phenolic Compounds on Human Skin

The bioactive substances incorporated as active ingredients in skin care formulations should be released from the vehicle or carrier to penetrate the stratum corneum and, subsequently, to deliver the cosmetic effects [10,15]. Under the experimental conditions adopted, the permeation profile of polyphenols from CS extract was evidenced after 8 h of testing. Only two polyphenols were identified in the permeated samples, namely ellagic acid (779.6 μg/g dw) and epicatechin (35.3 μg/g dw). Ellagic acid is a potent antioxidant and skin-whitening substance with photoprotective effects [16]. Also, catechin and derivatives are not only strong antioxidants, but also powerful ROS and RNS quenchers and anti-inflammatory agents [17]. In the present study, ellagic acid was the major phenolic compound quantified ($p < 0.05$), suggesting that this bioactive compound has substantial lipophilicity and smaller size than epidermis pores, allowing its easy permeation across the skin. However, the low amounts of ellagic acid after 8 h may indicate that a considerable portion was retained in the skin and only a small fraction passed through the skin layers into the receptor compartment. In a nutshell, the promising outcomes of skin permeation study revealed a slow penetration of phenolic compounds from CS extract into the skin, inducing a prolonged cosmetic effect [15]. Therefore, CS extract may be incorporated in cosmetic formulations as an active ingredient due to its ability to permeate the skin and pro-healthy properties of individual compounds. Interestingly, this is the first study that provides a careful assessment of the skin permeation of polyphenols-enriched CS extract.

4. Conclusions

The valorization of food by-products as cosmetic ingredients comprises a huge challenge for cosmetic industry. In this work, a chestnut by-product extracted by a sustainable extraction technique attested satisfactory anti-aging and antioxidant effects, as well as effective skin penetration of phenolic compounds and safeness on dermal cells, highlighting the importance of added value compounds present in food by-products for cosmetic use. Overall, the present study appraised a new cosmetic ingredient emphasizing its potential to be incorporated in cosmetic formulations as anti-aging ingredient, and also answering to the sustainable development goals of agenda 2030 of the United Nations and the European Regulation n.º 1223/2009.

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