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Complete List of Authors:	Rodrigues, Francisca; Requimte - Faculty of Pharmacy, University of Porto, ; Santos, Joana; Requimte - Faculty of Pharmacy, University of Porto, Pimentel, Filipa; Requimte - Faculty of Pharmacy, University of Porto, Braga, Nair; Requimte - Faculty of Pharmacy, University of Porto, Palmeira-de-Oliveira, Ana; CICS-UBI – Health Sciences Research Centre, University of Beira Interior, Oliveira, M.B.P.P.; Requimte - Faculty of Pharmacy, University of Porto,

Promising new applications for *Castanea sativa* shell: nutritional composition, antioxidant activity, amino-acids and vitamin E profile

Francisca Rodrigues[1,2], Joana Santos[1], Filipa B. Pimentel[1], Nair Braga[1], Ana Palmeira-de-Oliveira[3,4], M. Beatriz P.P. Oliveira [1,*]

1 - REQUIMTE, Department of Chemical Sciences, Faculty of Pharmacy, University of Porto, Rua de Jorge Viterbo Ferreira, 228, 4050-313 Porto, Portugal

2 - Fourmag Lda., Parque Industrial do Cruzeiro, Rua Nossa Senhora D'Ajuda, n.º 150, 4815 Moreira de Cónegos

3 - CICS-UBI – Health Sciences Research Centre, University of Beira Interior, 6200-506 Covilhã, Portugal

4 - Labfit-HPRD: Health Products Research and Development Lda, 6200-506 Covilhã, Portugal

*Corresponding author: M. Beatriz P.P. Oliveira, REQUIMTE, Department of Chemical Sciences, Faculty of Pharmacy, University of Porto, Rua de Jorge Viterbo Ferreira, 228, 4050-313 Porto, Portugal

Tel.: + 351 220428500; Fax: + 351 226093390

Email address: beatoliv@ff.up.pt

Abstract

The present study was aimed to assess the macronutrient composition and the amino acids and vitamin E profile of *Castanea sativa* shell from different Portuguese producing regions (Minho, Trás-os-Montes and Beira-Alta). The nutritional composition was similar for all samples, with a high moisture content and low fat amounts. Arginine and leucine were the predominant essential amino acids (EAA) accounting for 3.55 - 7.21% and 1.59 - 2.08%, respectively, for samples of the different production zones. All the shells presented high contents of vitamin E (481.5 mg/100 g sample, 962.8 mg/100 g sample and 567.5 mg/100 g sample, respectively, for Minho, Trás-os-Montes and Beira-Alta). The predominant vitamer was γ-tocopherol (670 mg/100 g sample for Trás-os-Montes). The antimicrobial and antioxidant activity of *C. sativa* shell were also determined. Trás-os-Montes extracts displayed the highest antioxidant activity (EC_{50} = 31.8 ± 1.3 μg/mL for DPPH; 8083.5 ± 164.8 μmol/mg db for FRAP). The total phenolic content (TPC) varied from 241.9 mg to 796.8 mg gallic acid equivalents (GAE)/g db sample, being the highest TPC obtained for Trás-os-Montes. The total flavonoid content (TFC) varied from 31.4 to 43.3 mg of catechin equivalents (CEQ)/g db sample. No antimicrobial activity was observed. Results showed the potentialities of *C. sativa* shell extracts.

Key-words: *Castanea sativa* by-products; shell; sustainability; antioxidants; vitamin E; amino acids

1. Introduction

Castanea sativa Mill. is a species of the Fagaceae family, abundant in south Europe and Asia. The fruits (chestnut) are an added value resource in producing countries, with an increased economic value due not only to its nutritional properties, but also to the beneficial health effects related with its consumption. In Portugal, chestnut trees are mainly used for nut production, representing a total area of about 35.000 ha, with a production of about 19.000 tons per year [1]. These fruits are a highly appreciated seasonal nut (autumn) in the Mediterranean countries [2], which can be consumed raw or cooked, although roasting or boiling are still the most common cooking procedures [3]. Chestnut processing and consumption generate high amounts of by-products, mainly shell, inner skin, outer skin and bur. These may represent a new challenge for the industry: to design innovative applications, answering simultaneously to sustainability concerns, while contributing to create new added value products and economic resources. Recently, different authors have suggested some potential applications of *C. sativa* shell as heavy metal adsorbent, as phenol substitutes in adhesive formulation or even as chrome substitutes in leather tanning [4]. However, only few studies have evaluated the antioxidant potential of this by-product [5].

Due to nutritional characteristics of the nut and for all the potentialities that *C. sativa* shell has shown so far, we believe that this by-product could also be used by other industries, such as pharmaceutical, food or cosmetic, as a natural source of bioactive compounds, generating more profits, reducing pollution costs and improving social, economic and environmental sustainability. Nevertheless, economic and environmental sustainability should be a priority for industries, encompassing the type of procedures, chemicals and solvents used to extract interesting compounds. For this reason, the extraction process should be designed as simple as possible, also considering the effective costs for industries [6]. In that sense, following other

previous studies that evaluated the combination of different solvents and temperatures to extract antioxidant compounds, the hydro-alcoholic solvent was chosen together with an extraction temperature of 50 °C [4, 6-8]. Besides, it is well known that climatic conditions such as temperature and humidity affect the antioxidant activity and the macronutrient composition of plant products. According to Dinis *et al.* leaf is the most flexible part of the chestnut tree in its response to environmental conditions [9]. The aim of this study was to evaluate the macronutrients, vitamin E and amino acids contents of *C. sativa* shells from three different producing regions in Portugal, namely, Minho, Trás-os-Montes and Beira-Alta. Also, the antioxidant and antimicrobial activity of hydro-alcoholic extracts of these samples were evaluated. To the best of our knowledge, no studies have been published regarding the macronutrients, amino acids, vitamin E content or even antimicrobial activity of this agro-industrial waste.

2. Materials and methods

2.1. Chemicals and reagents

For the macronutrients analysis all analytical grade reagents were purchased from Panreac (Barcelona, Spain) and Merck (Darmstadt, Germany). Tocopherols (α, β, ϒ and δ) and tocotrienols (α, β, ϒ and δ) were purchased from Calbiochem (La Jolla, California, USA) and tocol was obtained from Matreya Inc. (Pennsylvania, USA). Butylated hydroxytoluene (BHT) was from Aldrich (Madrid, Spain).

Ascorbic acid, 1,1-diphenyl-2-picrylhydrazyl ($DPPH^{\bullet}$) free radical, catechin, Folin–Ciocalteu's reagent, gallic acid, iodine, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid, glycerol, a water-soluble derivative of vitamin E (Trolox) and α-tocopherol, were all purchased from Sigma - Aldrich (Steinheim, Germany). Ethanol reagent grade, sodium acetate, sodium

carbonate decahydrate, sodium nitrite, aluminium chloride and sodium hydroxide were purchased from Merck (Darmstadt, Germany).

HPLC-grade acetonitrile and 1,4-dioxane were from Fluka (Madrid, Spain). HPLC grade n-hexane was from Merck (Darmstad, Germany). Purified water was obtained from a Milli-Q water purification system (Millipore, Bedford, MA, USA).

Vitek-2 identification cards were from Biomerieux (Crappone, France). Brain Heart Infusion, Nutrient agar, Sabouraud dextrose agar and Mueller Hinton broth were purchased from Difco Laboratories (USA). RPMI was from Biochrom (Berlin, Germany).

2.2. Samples

C. sativa fruits were collected during September 2013 in three different regions of Portugal, namely, Minho, Trás-os-Montes and Beira-Alta, and dried at room temperature for 4 weeks. Samples were prepared simulating the traditional processing of roasting. Chestnuts were baked in the oven (Tecnogás, Portugal) at 240 ° C for 35 minutes. Subsequently, shell was separated from chestnut fruit, milled at particle size of approximately 0.1 mm using an A11 basic analysis mill (IKA Wearke, Staufen, Germany) and stored in plastic tubes at 4°C until the extracts preparation.

2.3. Preparation of hydro-alcoholic extracts

Powdered samples (1 g) were submitted to solvent extraction by maceration with 20 mL of ethanol: water (1:1) at 40 °C for 30 minutes (Mirac, Thermolyne, USA). Extracts were filtered through Whatman No. 1 filter paper, concentrated under vacuum at 37 °C and kept under refrigeration (4 °C) prior to use.

2.4. Macronutrients analysis

Moisture content was instrumentally determined using an infrared moisture analyzer (SMO 01, Scaltec Instruments, Germany). The ash content was determined by incinerating the sample in a muffle furnace at 550 °C, according to 923.03 method [10]. Protein content (factor 6.25) was determined using the Kjeldahl procedure [11]. Total fat was determined by Soxhlet [12]. Total carbohydrate content was determined by difference [13]. Analyses were performed in triplicate and results are expressed as g/100 g.

2.5. Total vitamin E and vitamers quantification

Lipid fraction for vitamin E quantification was obtained by Soxhlet extraction with petroleum ether (2.5 h). The chromatographic analysis was carried out in an HPLC integrated system equipped with AS-2057 automated injector, a PU-2089 pump and a MD-2018 multi-wavelength diode array detector (DAD) coupled to a fluorescence detector FP-2020 (Jasco, Japan), programmed for excitation at 290 and emission at 330 nm. The chromatographic separation of the compounds was achieved on a normal phase Supelcosil™ LC-SI (3 µm; 75 X 3.0 mm; Supelco, Bellefonte, PA, USA) according to Alves *et al.* [14]. Chromatographic data were analyzed using JASCO-Chrom NAV Chromatography Software (Jasco, Japan). Results are expressed in mg/100 g of sample. Analyses were performed in triplicate.

2.6. Amino acids quantification

Amino acids (Histidine (His), Arginine (Arg), Serine (Ser), Aspartic acid + Glutamic Acid (Asp + Glu), Threonine (Thr), Glycine (Gly), Alanine (Ala), Proline (Pro), Valine + Methionine (Val + Met), Phenylalanine (Phe), Isoleucine (Ile), Leucine (Leu), Ornithine (Orn), Lysine (Lys) and Tyrosine (Tyr)) were analyzed by reversed-phase HPLC with fluorescence detection after submitting samples to an acidic hydrolysis (HCl 6 mol/L, 110 °C, 24 h) [15-16] and derivatization with dansyl chloride [17-18]. The protein fraction obtained from Kjeldahl procedure was used.

The chromatographic analysis was carried out in an HPLC integrated system equipped with an AS-950 automated injector, a PU-980 pump, a CO-2060 Plus oven, and an FP-920 fluorescence detector (Jasco, Japan) programmed for excitation at 335 nm and emission at 514 nm. The compounds separation was achieved in a C18 RP Luna column (4.6 x 250 mm, 5 µm; Supelco, Bellefonte, PA, USA) from Phenomenex (Torrance, CA, USA), according to Pimentel *et al.* [18]. Chromatographic data were analyzed using the JASCO-Chrom NAV Chromatography Software (Jasco, Japan). The amino acids were identified by retention time comparison with authentic standards. Quantification was carried out on the basis of the internal standard method. Results are expressed in mg/ 100 g of sample. Analyses were performed in triplicate.

2.7. Determination of total phenolic content

Total phenolic content (TPC) was determined spectrophotometrically according to the Folin–Ciocalteu procedure [19], with minor modifications [20]. Briefly, 500 µL of extract was mixed with 2.5 mL of Folin–Ciocalteu reagent (10 x dilution) and allowed to react for 5 min. Then 2.5 mL of Na_2CO_3 7.5 % solution was added and allowed to stand for 15 min at 45 °C and 30 min at room temperature, before the absorbance being determined at 765 nm using a Synergy HT Microplate Reader (BioTek Instruments, Inc., Winooski, VT, USA). A calibration curve for the standard gallic acid was used to obtain a correlation between sample absorbance and standard concentration (linearity range = 5 - 100 µg/mL, $R^2 > 0.998$). The total polyphenol content (TPC) of the extracts was expressed as mg of gallic acid equivalents (GAE) per gram of plant material on dry basis (db).

2.8. Determination of total flavonoid content

Total flavonoid content (TFC) was determined by a colorimetric assay based on the formation of flavonoid-aluminum compound according to Rodrigues *et al.* [6]. Briefly, 1 mL of a diluted extract was mixed with 4 mL of ultrapure water and 300 µL of 5 % (*w/v*) $NaNO_2$ solution. After

5 min, 300 µL of 10% (*w/v*) g/100 mL $AlCl_3$ solution were spiked, and after 1 min, 2 mL of 1 mol/L NaOH and 2.4 mL of ultrapure water were also added. The absorbance was read at 510 nm using the Synergy HT Microplate Reader. Catechin was used as reference to plot the standard curve (linearity range = 0 - 400 µg/mL, R^2 > 0.999). Total flavonoid concentration (TFC) was expressed as milligrams of catechin equivalents (CAE) per gram of plant material on db.

2.9. In vitro antioxidant activity

The antioxidant activity of the samples was evaluated by $DPPH^{\bullet}$ radical-scavenging activity and ferric reducing antioxidant power (FRAP) as detailed in the following sections.

2.9.1 DPPH free radical scavenging assay

The reaction mixture was made directly on a 96 wells plate and consisted of a solution of different sample concentrations (30 µL) and methanol solution (270 µL) containing DPPH radicals (6 x 10^{-5} mol/L), in each well. The mixture was left to stand for 30 min in the dark. The reduction of the DPPH radical was determined by measuring the absorption at 517 nm [21]. The radical scavenging activity (RSA) was calculated as a percentage of DPPH discolouration using the equation: % RSA = [(ADPPH–AS)/ADPPH] × 100, where AS is the absorbance of the solution when the sample extract has been added at a particular level, and ADPPH is the absorbance of the DPPH solution [22]. The extract concentration providing 50% of radicals scavenging activity (EC_{50}) was calculated from the graph of RSA percentage against extract concentration. BHA and α-tocopherol were used as standards.

2.9.2 Ferric reducing antioxidant power (FRAP) assay

FRAP assay was carried out by the method of Benzie and Strain [23], with minor modifications. The method is based on the reduction of a ferric 2,4,6-tripyridyl-s-triazine complex (Fe^{3+}-TPTZ) to the ferrous form (Fe^{2+}-TPTZ). An aliquot (90 µL) of an extract (with appropriate dilution, if

191 necessary) was added to 2.7 mL of FRAP reagent (10 parts of 300 mM sodium acetate buffer at
192 pH 3.6, 1 part of 10 mM TPTZ solution and 1 part of 20 mM $FeCl_3 \cdot 6H_2O$ solution) and the
193 reaction mixture was incubated at 37 °C. The increase in absorbance at 592 nm was measured
194 after 30 min. Solutions of known Fe (II) concentrations ($FeSO_4$-$7H_2O$) were used for calibration.
195 A calibration curve was prepared with ferrous sulphate (linearity range: 150 - 2000 µM, R^2 >
196 0.996), and the results were expressed as ferrous sulphate equivalent per gram of plant
197 material on db.

198 **2.10. Antimicrobial assay**

199 **2.10.1 Microorganisms strains**

200 Eight microorganisms (seven bacteria and one yeast) were included in the study. Both
201 American Type Culture Collection (ATCC) and clinical isolates were selected, corresponding to
202 *Staphylococcus aureus* (ATCC 6538 and a clinical isolate), *S. epidermidis* (clinical isolate),
203 *Escherichia coli* (ATCC 1576 and a clinical isolate), *Klebsiella peumoniae* (ATCC 4352),
204 *Pseudomonas aeruginosa* (ATCC 9027) and *Candida albicans* (ATCC 10231). Clinical isolates
205 were identified to species-level with Vitek-2 identification cards and all strains were kept
206 frozen in Brain Heart Infusion with 20% glycerol at -70° C until testing. For each experiment
207 microorganisms were subcultured twice in Nutrient agar for bacteria and Sabouraud dextrose
208 agar for *C. albicans*, in order to access culture viability.

209 **2.10.2 MIC by broth dilution assay**

210 The antibacterial activity was tested according to the Clinical and Laboratory Standards
211 Institute (CLSI) M7-A6 micromethod and the antifungal activity was tested according to M27-
212 A3 micromethod from the same protocol, after 48 h of incubation at 37 °C [24-25]. Briefly, two-
213 fold serial dilutions of extracts were performed in Mueller Hinton broth for bacteria and in
214 RPMI for yeast. Concentrations ranging from 10 µg/mL to 1000 µg/mL were tested.

Microorganism's growth was visually compared for each concentration with the growth control (without plant extract). Minimal inhibitory concentration (MIC) was defined as the lowest extract concentration able to completely inhibit microorganism growth, corresponding to 100% MIC value. All determinations were performed in duplicate for each assay and three independent experiments were run with concordant results.

2.11. Statistical analysis of data

Data were reported as mean ± standard deviation of at least triplicate experiments. Statistical analysis of the results was performed with SPSS 22.0 (SPSS Inc., Chicago, IL, USA). One-way ANOVA was used to investigate the differences between different samples for all assays. Post hoc comparisons of the means were performed according to Tukey's HSD test. In all cases, $P < 0.05$ was accepted as denoting significance.

3. Results and discussion

3.1 *Castanea sativa* shell

3.1.1 Macronutrient composition

Through the assessment of several physical and chemical parameters it is possible to infer the state of maturation and conservation, as well as the nutritional and commercial values of fruits and their by-products [26]. To the best of our knowledge, this is the first study about the ash, moisture, fat, carbohydrates and protein content of *C. sativa* shell, showing promising results for the valorization of this by-product. Table 1 summarizes the macronutrient composition determined in this work.

Insert Table 1

Moisture is one of the most representative fractions in all samples, with statistical differences between them. Shell, as a chestnut protection, is expected to have lower moisture content in relation to the fruit. The difference between samples could result from the different climatic conditions of each production region. For example, Minho region presents the highest precipitation levels which might lead to the higher moisture value observed.

Regarding protein content, it ranged between 2.77 and 3.13 g/100 g, respectively, for Trás-os-Montes and Minho. Comparing with chestnut, the content was lower, as the fruit normally presents a 6.0 to 8.6% of protein in their composition. The ash values found in the shell samples were also slightly different from contents described for chestnuts [27-28]. Concerning the fat content, the chestnut shell from Trás-os-Montes region presented the highest value, statistically different from the other regions. The value found was also lower than the one normally described in the fruit [29]. The lower protein, ash and fat content in relation to the values described for chestnut fruit could be expected due to the higher presence of others constituents such as dietary fiber, normally composed by cellulose, hemicellulose and lignin, common in a higher proportion in fruit shells.

3.1.2 Amino acids content

The amino acids content evaluated in the chestnuts shell is shown in Table 2.

Insert Table 2

Arginine (Arg) and Leucine (Leu) were the predominant essential amino acids (EAA) in the chestnut shell samples accounting for 355 – 721/100 g sample and 159 – 20/ 100 g sample, respectively. Tyrosine (Tyr), phenylalanine (Phe) and threonine (Thr) were present, but in lower amounts, 14 - 20, 63 - 91 and 74 - 125, respectively. Histidine (His) was not detected in any of the analyzed samples. Glutamic and aspartic acid (Asp + Glu) where, in their turns, the predominant non-essential amino acids (NEA) detected.

A balanced protein intake is important to maintain a healthy diet and general wellbeing. The development of new supplements rich in amino acids is of major interest for industry and consumers. According to the obtained results, that showed a balanced profile of amino acid in the chestnut shell, the use of this by-product as a possible and cheap source of amino acids should not be neglected.

3.1.3 Total Vitamin E and vitamers quantification

Vitamin E is the major lipid-soluble antioxidant in the cell antioxidant defence system and is exclusively obtained from the diet. Vitamin E is an essential nutrient, occurring in eight main chemical forms of related structure, tocopherols (α-, β-, γ-, and δ-) and tocotrienols (α-, β-, γ-, and δ-). α-Tocopherol is the most common form found in photosynthetic tissues [30]. Vitamin E is known as one of the most powerful fat-soluble antioxidant, helping in the promotion of cardiovascular health, enhancement of the immune system function, aid in skin repair and protecting cell membranes from damage caused by free radicals [31]. Table 3 summarizes the Vitamin E profile and total content of *C. sativa* shell.

Insert Table 3

Regarding the total vitamin E content, there were statistical differences observed between regions. Trás-os-Montes presented the highest value and Minho the lowest one, with values ranging between 962.8 mg/100 g and 481.5 mg/100 g sample, respectively. Moreover, a considerable difference between the Vitamin E profiles in shells from different regions was found. This variation might be due to climatic differences as tocopherol acts essentially as antioxidant compound, being affected by different conditions such as temperature or humidity. Probably is a response of the plant to environmental stress.

From a qualitative point of view, shells present a vitamin E profile quite similar to chestnut [32-33]. Likewise to what is described for the chestnut fruit, ϒ-tocopherol was the prevailing

288 vitamer detected in shells, accounting for more than half of the total profile determined for all
289 studied samples. Interestingly, this vitamer is often present in trace amounts in other natural
290 products.

291 **3.2 *Castanea sativa* hydro-alcoholic extracts**

292 The extraction yield (expressed as w/w percentages) for chestnut shell from Trás-os-Montes
293 and Minho are globally similar, varying from 12.57 ± 0.01 % to 13.67± 0.02%, which can be
294 accepted as satisfactory [27, 34]. In the shell samples collected in Beira-Alta region, the extraction
295 yield was 4.10 ± 0.10%, considerably lower than those obtained for other samples. Indeed,
296 comparing the results with the ones described by Barreira *et al.* (2008), the extraction yield
297 obtained was inferior, what could be explained by the higher polarity of water, the extractor
298 solvent used by those authors [38].
299

300 **3.2.1 Total phenolic, total flavonoid content and antioxidant activity**

301 Table 4 presents the total phenolic and flavonoid contents, the EC_{50} values and the antioxidant
302 activity based on FRAP assay obtained for all shell extracts.

303 Insert Table 4

304 Among all extracts analyzed, the chestnut shell from Trás-os-Montes presented the highest
305 value (796.80 ± 1.07 mg GAE/g db), followed by Minho and Beira-Alta. The significant
306 differences observed can be explained on the basis of environmental conditions, as all samples
307 were treated in the same away. Minho and Beira-Alta are rainfall regions, compared to Trás-
308 os-Montes, which is more dry and arid. These factors can affect the environmental and
309 ecological quality of the soil, such as water regulation and biodiversity. The temperature rise,
310 associated with conditions of greater dryness, may also have a direct effect on organic matter
311 decline, due to a lower production of biomass and mineralization. The variation observed

312 between the extracts from the different regions may reflect that. Indeed, organic compounds
313 and the development of defenses to combat stress caused by situations of unfavorable
314 weather conditions, may enhance biological differences in composition between the three
315 regions, justifying their antioxidant properties variations [35].

316 The results obtained for the chestnut shell extracts from Trás-os-Montes were in accordance
317 with the ones presented by Barreira *et al.* (2010), who also evaluated the TPC of chestnut shell
318 of different cultivars from the same region, using water as solvent extractor [36] . In turn, the TPC
319 values presented by Vázquez *et al.* (2008), that extracted chestnut shell with water and
320 different organic solvents, were lower [5]. In this sense, it is possible to infer that the use of
321 ethanol and water as extraction solvents achieves a reasonable content of TPC in the extract,
322 and simultaneously comply with sustainability concept as it uses green chemicals. The values
323 obtained can also be compared to other *C. sativa* by-products, like chestnut bur, for which
324 considerably lower TPC values have been reported. This reinforces the potentialities of
325 chestnut shell as a source of bioactive compounds[37]. The obtained TPC content is considerable,
326 revealing the great potential of the assayed by-products extracts as a new source of
327 antioxidant compounds [6-7].

328 Flavonoids are an important group of phenolic compounds, contributing to the antioxidant
329 activity of plant products. In this assay, the highest TFC values were found in sample extracts
330 collected in Trás-os-Montes. Barreira *et al.* (2008) found a TFC slightly higher for shell extracts
331 from Trás-os-Montes that could be explained by natural seasonal variations and also by the
332 use of different solvents [38].

333 Regarding EC_{50} values, Trás-os-Montes shell extracts also presented the best results (31.80 ±
334 1.13 μg/ml), however no statistic differences ($P > 0.05$) were observed between the different
335 regions. Once more, the values found were slight inferior to the values reported by Barreira *et*

al. (2010), whose EC_{50} values for chestnut shell extracts ranged between 82.41 and 159.99 µg/mL for the same sample [36]. Regarding the evaluation of the antioxidant capacity of extracts based on FRAP assay, the obtained results showed a high antioxidant activity of all regions tested, being Trás-os-Montes again the best (8083.50 ± 164.80 µmol of ferrous sulphate/ g db). The values were higher than those reported in other works [6-7, 36], strengthening once more the potential of the chestnut shell. The antioxidant capacity of plant products is usually related to a high phenolic content, and also to a higher vitamin E contents. In this work, the highest antioxidant activity was registered for the extracts that simultaneously presented the higher phenolic and flavonoid contents (samples from Trás-os-Montes), which reinforces the importance of these compounds to the antioxidant activity.

3.2.2 Antimicrobial activity

The minimum inhibitory concentrations (MICs) for the active plant extracts were determined using modified methods of the CLSI against microorganisms selected to cover different Gram-positive bacteria (*S. aureus* and *S. epidermidis*), Gram negative bacteria (*E. coli*, *K. pneum*oniae, *P. aeruginosa*) and yeasts (*C. albicans*), that normally inhabit or proliferate in the human body. The microorganism's strains were selected considering possible future application of this food by-product in different industries such as pharmaceutical or cosmetic. Results did not reveal inhibitory activity against any of the microorganisms tested.

4. Conclusion

The use of *C. sativa* shell as a source of functional compounds and their application for different purposes, such as pharmaceutical, food supplementation or even cosmetic, is a promising field which requires interdisciplinary research, taking into account sustainability concerns.

In this work, *Castanea sativa* shell from different Portuguese regions (Minho, Trás-os-Montes and Beira-Alta) and their hydro-alcoholic extracts were analyzed and compared. Shell extracts from Trás-os-Montes presented the highest TPC, TFC, and antioxidant capacity confirmed by their highest value in the FRAP assay and lower EC_{50}. Regarding the amino acid content, all extracts are rich in essential amino acids. The vitamin E results were also very promising. However, none of the extracts presented antimicrobial activity. The results of this work, thus, suggested that *C. sativa* shell extracts, especially from Trás-os-Montes, could be a new promising active ingredient with potential applications, such as in food supplements. Further investigations are now needed to establish the citotoxicity of these extracts in different cell lines, such as Caco-2 cell model or fibroblasts, in order to test their incorporation in different food and/or cosmetic products.

Conflict of interest

The authors declare no conflict of interest. This article does not contain any studies with human or animal subjects.

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References

1. Portuguese National Institute of Statistics IP. ISSN 0079-4139; ISBN 978-989-25-0198-7 2013, 2013.
2. B. R. Cruz, A. S. Abraão, A. M. Lemos and F. M. Nunes, *Carbohydr Polym*, 2013, **94**, 594-602.
3. N. Braga, F. Rodrigues and M. B.P.P.Oliveira, *Nat Prod Res*, 2014, 1-18.
4. G. Vázquez, J. González-Alvarez, J. Santos, M. S. Freire and G. Antorrena, *Ind Crop Prod*, 2009, **29**, 364-370.
5. G. Vázquez, E. Fontenla, J. Santos, M. S. Freire, J. González-Álvarez and G. Antorrena, *Ind Crop Prod*, 2008, **28**, 279-285.
6. F. Rodrigues, A. Palmeira-de-Oliveira, J. das Neves, B. Sarmento, M. H. Amaral and M. B. P.P.Oliveira, *Ind Crop Prod*, 2013, **49**, 634-644.
7. F. Rodrigues, A. Palmeira-de-Oliveira, J. das Neves, B. Sarmento, M. H. Amaral and M. B. P. P. Oliveira, *Pharm Biol*, 2014, **4**, 1-9.
8. A. S. G. Costa, R. C. Alves, A. F. Vinha, S. V. P. Barreira, M. A. Nunes, L. M. Cunha and M. B. P. P. Oliveira, *Ind Crop Prod*, 2014, **53**, 350-357.
9. L. T. Dinis, F. Peixoto, T. Pinto, R. Costa, R. N. Bennett and J. Gomes-Laranjo, *Environ Exp Bot*, 2011, **70**, 110-120.
10. AOAC, in *Official methods of analysis of AOAC International*, editor W. Horwitz, Gaithersburg. USA, 17th edition, 2000, chapter 32, p. 2.
11. AOAC, Ash of flour (923.03) in *Official methods of analysis of AOAC International*, editor W. Horwitz, Gaithersburg. USA, 17th edition, 2000.
12. AOAC, Fat (crude) (920.39) in *Official methods of analysis of AOAC International*, editor W. Horwitz, Gaithersburg. USA, 17th edition, 2000.
13. K. Tontisirin, W. C. MacLean and P. Warwick, Food and Agriculture Organization of the United Nation editor, Rome, Italy, 2003, pp. 1-66.
14. R. C. Alves, S. Casal and M. B. P. P. Oliveira, *Food Sci Technol Int*, 2009, **15**, 57-63.
15. M. Fountoulakis and H.-W. Lahm, *J Chromatogr A*, 1998, **826**, 109-134.
16. A. M. G. Paramás, J. A. G. Bárez, C. C. Marcos, R. J. García-Villanova and J. S. Sánchez, *Food Chem*, 2006, **95**, 148-156.
17. J. L. Navarro, M. Aristoy and L. Izquierdo, *Rev Agroquim Tecnol*, 1984, **24**, 85-93.
18. F. B. Pimentel, R. C. Alves, A. S. G. Costa, D. Torres, M. F. Almeida and M. B. P. P. Oliveira, *Food Chem*, 2014, **149**, 144-150.
19. V. L. Singleton and J. A. J. Rossi, *Am J Enol Vitic*, 1965, **16**, 144-158.
20. R. Alves, A. Costa, M. Jerez, S. Casal, J. Sineiro, M. Núñez and M. B. P. P. Oliveira, *J Agr Food Chem*, 2010, **58**, 12221-12229.
21. R. Guimarães, L. Barros, J. C. M. Barreira, M. J. Sousa, A. M. Carvalho and I. C. F. R. Ferreira, *Food Chem Toxicol*, 2010, **48**, 99-106.
22. L. Barros, P. Baptista and I. C. F. R. Ferreira, *Food Chem Toxicol*, 2007, **45**, 1731-1737.
23. I. F. F. Benzie and J. J. Strain, in *Methods in Enzymology*, In: Lester P, editor. Methods in Enzymology: Academic Press, 1999, 299, 15-27.
24. C.L.S.I. Clinical Laboratory Standards Institute,NCCLS editor, Wayne, Pennsylvania, 2008.
25. M. Wikler, D. Low, F. Cockerill, D. Sheehan, W. Craig, F. Tenover, M. Dudley, J. Turnidge, G. Eliopoulos, M. Weinstein, D. Hecht, B. Zimmer, J. Hindler, M. Ferraro and J. Swenson, NCCLS, Wayne, Pennsylvania., 2003, 26.
26. A. F. Vinha, R. C. Alves, S. V. P. Barreira, A. Castro, A. S. G. Costa and M. B. P. P. Oliveira, *LWT - Food Sci Technol*, 2014, **55**, 197-202.
27. O. Borges, B. Gonçalves, J. L. S. de Carvalho, P. Correia and A. P. Silva, *Food Chem*, 2008, **106**, 976-984.

28. S. Pereira-Lorenzo, A. M. Ramos-Cabrer, M. B. Díaz-Hernández, M. Ciordia-Ara and D. Ríos-Mesa, *Sci Hort*, 2006, **107**, 306-314.
29. B. Gonçalves, O. Borges, H. S. Costa, R. Bennett, M. Santos and A. P. Silva, *Food Chem*, 2010, **122**, 154-160.
30. M. Hasanuzzaman, K. Nahar and M. Fujita, in *Emerging Technologies and Management of Crop Stress Tolerance*, P. Ahmad and S. Rasool editors, Academic Press, San Diego, 2014, 267-289.
31. O. Guralp, *Maturitas*, 2014, **79**, 476-480.
32. M. D. Zlatanov, G. A. Antova, M. J. Angelova-Romova and O. T. Teneva, *J Sci Food Agric*, 2013, **93**, 661-666.
33. J. C. M. Barreira, R. C. Alves, S. Casal, I. C. F. R. Ferreira, M. B. P. P. Oliveira and J. A. Pereira, *J Agric Food Chem*, 2009, **57**, 5524-5528.
34. J. De La Montaña Míguelez, M. Míguez Bernárdez and J. M. García Queijeiro, *Food Chem*, 2004, **84**, 401-404.
35. Strategies for adaptation of agriculture and forestry to climate change. In: Ministry of Agriculture S, Environment and Territory, editor. Lisbon: Portuguese Government, 2013, 1-88.
36. J.C.M. Barreira, I. C. F. R. Ferreira, M. B. P. P. Oliveira and J. A. Pereira, *Food Sci Technol Int*, 2010, **16**, 209-216.
37. E. M. Balboa, M. L. Soto, D. R. Nogueira, N. González-López, E. Conde, A. Moure, M. P. Vinardell, M. Mitjans and H. Domínguez, *Ind Crop Prod*, 2014, **58**, 104-110.
38. J. C. M. Barreira, I. C. F. R. Ferreira, M. B.P. P. Oliveira and J. A. Pereira, *Food Chem*, 2008, **107**, 1106-1113.

Table Captions

Table 1 – Nutritional composition of the *C. sativa* shell from different production regions (mean ± standard deviation). Different letters (a, b, c) in the same line indicate significant differences between mean values ($P < 0.05$).

Table 2 - Amino acids content (mg/100 g sample) of the shell of chestnut samples from different production regions (mean ± standard deviation). Different letters (a, b, c) in the same line indicate significant differences between mean values ($P < 0.05$). nd – not detectable.

Table 3 – Vitamin E total content and vitamers (mg / 100 g sample) of chestnut shell from different regions (mean ± standard deviation). Different letters (a, b, c) in the same line indicate significant differences between mean values ($P < 0.05$). nd – not detectable.

Table 4 – Total polyphenol content (TPC), Total flavonoid content (TFC), Radical scavenging activity (RSA) EC_{50} values and antioxidant activities based on their abilities to reduce ferric iron (Fe^{3+}) to ferrous iron (Fe^{2+}) in the three hydro-alcoholic shell extracts. Values are expressed as means ± standard deviation (n = 6). GAE, gallic acid equivalents. CEQ, cathechin equivalents. Different letters (a, b, c) in the same column indicate significant differences between mean values ($P < 0.05$).

Table 1

	Region		
	Minho	**Trás-os-Montes**	**Beira-Alta**
Moisture (g/100g db)	38.61 ±1.78[a]	21.29±1.56[c]	26.21±0.40[b]
Ash (g/100g db)	1.60 ± 0.06	1.36 ± 0.04	1.08 ± 0.04
Proteins (g/100g db)	3.13 ± 0.10	2.77 ± 0.00	2.89 ± 0.17
Fat (g/100g db)	0.15 ± 0.00[b]	0.52 ± 0.05[a]	0.23 ± 0.02[b]
Carbohydrates (g/100 g db)	56.51	74.06	70.41

Table 2

	Zone		
Amino acids	**Minho**	**Trás-os-Montes**	**Beira-Alta**
Essential amino acids (EAA)			
His	nd	nd	nd
Ile	132 ± 10^{a}	143 ± 4^{a}	99 ± 10^{b}
Leu	208 ± 16^{b}	246 ± 10^{a}	159 ± 15^{c}
Lys	132 ± 11^{b}	156 ± 4^{a}	109 ± 1^{c}
Phe	79 ± 7^{b}	91 ± 4^{a}	63 ± 6^{b}
Tyr	20 ± 2^{a}	19 ± 1^{a}	14 ± 7^{b}
Thr	101 ± 5^{b}	125 ± 9^{a}	74 ± 6^{c}
Arg	721 ± 71^{a}	715 ± 78^{a}	355 ± 19^{b}
Val + Met	51 ± 4^{a}	58 ± 1^{a}	38 ± 4^{b}
Non-essential amino acids (NEAA)			
Ala	84 ± 6^{b}	95 ± 4^{a}	51 ± 4^{c}
Ser	121 ± 12^{b}	160 ± 13^{a}	106 ± 4^{b}
Asp + Glu	403 ± 38^{b}	484 ± 32^{a}	268 ± 12^{c}
Gly	71 ± 4^{b}	79 ± 4^{a}	51 ± 1^{c}
Pro	182 ± 15^{b}	241 ± 9^{a}	185 ± 22^{b}
Orn	9 ± 1^{a}	1 ± 1^{b}	2 ± 1^{b}

Table 3

Compound analyzed	Region		
	Minho	Trás-os-Montes	Beira-Alta
α-tocopherol	65.94 ± 2.83[c]	190.8 ± 0.00[a]	162.1 ± 0.90[b]
β-tocopherol	12.00 ± 0.10[b]	14.2 ± 0.30[a]	6.7 ± 0.10[c]
δ-tocopherol	29.86 ± 0.83[a]	69.3 ± 0.70[b]	33.6 ± 0.40[b]
ϒ-tocopherol	228.58 ± 0.85[c]	670.4 ± 0.20[a]	356.5 ± 1.70[b]
α-tocotrienol	19.37 ± 0.28[a]	8.6 ± 0.10[b]	4.3 ± 0.00[c]
β-tocotrienol	34.56 ± 2.55	nd	nd
ϒ-tocotrienol	67.79 ± 0.90	nd	nd
δ -tocotrienol	23.35 ± 1.18[a]	9.6 ± 0.10[b]	4.3 ± 0.10[c]
Total	481.5 ± 1.19	962.8 ± 0.23	567.5 ± 0.53

Table 4

Region	TPC (mg GAE/ g db)	TFC (mg CEQ / g db)	EC_{50} (µg/mL)	FRAP (µmol of ferrous sulphate/g db)
Minho	241.90 ± 0.26[b]	31.38 ± 2.88[c]	37.61 ± 5.08[a]	6891.20 ± 355.00[b]
Trás-os-Montes	796.80 ± 1.07[a]	43.33 ± 1.00[a]	31.80 ± 1.13[a]	8083.50± 164.80[a]
Beira-Alta	143.00 ± 0.57[c]	37.95 ± 1.52[b]	36.81 ± 4.55[a]	6008.70± 138.60[c]

Castanea sativa shell

Sustainability / Re-Use

- Macronutrients analysis
- Vitamin E and vitamers
- Amino acids
- Antioxidant activity
- Antimicrobial activity