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Exploring the Impact of In Vitro Gastrointestinal Digestion in the Bioaccessibility of Phenolic-Rich Chestnut Shells: A Preliminary Study

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Abstract: Chestnut shells (CS), the principal by-product of the chestnut processing industry, contain high concentrations of flavonoids and other polyphenols with huge interest for the nutraceuticals field. Nonetheless, the bioaccessibility and bioactivity of phytochemicals can be influenced by their digestibility, making it imperative to evaluate these activities prior to application of CS as a nutraceutical ingredient. This work aims to appraise the effects of in vitro simulated gastrointestinal digestion on the bioaccessibility, bioactivity, and metabolic profiling of CS. An increase in the total phenolic and flavonoid contents, antioxidant/antiradical properties, radical scavenging capacity, and inhibition on acetylcholinesterase activity was evidenced during in vitro simulated digestion. Metabolomic profiling by LC-ESI-LTQ-Orbitrap-MS revealed changes during the simulated digestion, particularly in phenolic compounds (46% of total compounds annotated), lipids (22%), phenylpropanoids (9%), organic acids (7%), carbohydrates (5%), nucleosides (5%), amino acids (4%), and alcohols (1%). Phenolic acids (gallic acid, syringic acid, and hydroxyphenylacetic acid) and flavonoids (epicatechin) were the major polyphenolic classes identified. The heatmap-positive correlations highlighted that the bioactivity of CS is closely related to the phenolic compounds and their bioaccessibility. These findings suggest the reuse of CS as a potential nutraceutical ingredient with antioxidant and neuroprotective effects, encouraging the use of appropriate extraction and/or encapsulation techniques to enhance the bioaccessibility of phenolic compounds.

Keywords: *Castanea sativa*; simulated digestion; antioxidant activity; high-resolution mass spectrometry; green chemistry; sustainability

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1. Introduction

Over the past decade, different initiatives have been implemented worldwide to address food safety, sustainable development, human health, and environmental preservation, as nations adopt preventive measures towards a circular economy [1]. The reuse of by-products generated by agro-industries as added-value feedstocks for the extraction of bioactive molecules encompasses a global trend to design more sustainable and competitive businesses [2]. Within this scenario, agro-industrial residues have been widely explored [3–9].

Castanea sativa is a European chestnut tree from the Fagaceae family with an inestimable socioeconomic value in rural areas, owing to the nuts' nutritional and sensory properties [10]. Chestnut production reached more than 5.1 million metric tons in 2021–2022, representing a 50% increase when compared to the past 10 years [11]. According to the Food and Agriculture Organization (FAO), coconuts and chestnuts contributed 35% and 25% of global nut production, respectively [11].

The rising demand for healthy snacks has driven an increase in their global production [1,12,13]. The production of industrial chestnut processing constitutes about 20% shells, representing the principal chestnut by-product without an effective commercial value [14]. Although chestnut fruits have been widely used in the development of food products (i.e., flour, jam, marron glacé, and purée), their by-products have no profitable applications, currently being only managed as a renewable resource for composting [1,10]. Recent studies proposed different industrial applications of chestnut shells (CS), considering their nutritional value, phytochemical composition, and biological activities [7,9,15–19]. Their wealth of bioactive compounds are nutritionally and industrially relevant with promising uses highlighted in the food, nutraceutical, and cosmetic sectors [7,9,15–19]. Recently, Pinto et al. [12,20] proposed the development of functional cookies incorporating a phenolic-rich CS extract as a new healthy snack intended for the prevention/co-therapy of oxidative stress-mediated disorders. In addition, the fortified cookies were effective in the delivery of antioxidant molecules, even after in vitro simulated digestion, providing mild hypoglycemic and neuroprotective effects, possibly ascribed to the phenolic compounds delivered by the CS extract [12]. Nevertheless, it is imperative to comprehend the bioaccessibility and bioactivity of polyphenols in CS and not only in the final product, because these properties may be attributed, even to a lower extent, to other ingredients used in the formulation.

In vitro digestion has been extensively described as a useful approach to predict the gastrointestinal behavior and bioaccessibility of a food ingredient or product with several benefits over human trials, including lower cost, faster, efficiency, and no ethical restrictions [21]. Some studies have already highlighted the value of other nuts and seeds for nutrition and health, considering macronutrients (e.g., proteins, carbohydrates, and fat) and micronutrients (e.g., phenolic compounds, vitamins, and minerals) [1,7,9,14,22,23]. Notwithstanding, just few studies have provided a comprehensive evaluation of the digestive process in the bioaccessibility and bioactivity of nuts [24,25]. Therefore, the present study explores the effects of in vitro simulated gastrointestinal digestion in the bioaccessibility and bioactivity of soluble phenolic compounds from CS (that we have been targeting for valorization in the food and nutraceutical industries), evaluating the total phenolic and flavonoid contents, antioxidant/antiradical properties, reactive oxygen and nitrogen species (ROS and RNS, respectively) scavenging efficiency, acetylcholinesterase (AChE) activity inhibition, and metabolomic profiling of targeted phenolic compounds before and after in vitro digestion. Although several studies have evaluated the phytochemical composition and biological properties of CS, this study provides the first comprehensive assessment of CS bioactivity post-in vitro digestion, elucidating its metabolization and biotransformation in each digestion phase as well as its potential biological effects on human health.

2. Materials and Methods

2.1. Chemicals

All chemicals and standards were of analytical reagent grade and supplied by commercial sources. In metabolomic analyses, the chemicals acquired and used were as follows: 2,5-dihydroxybenzoic acid, 2,6-dihydroxybenzoic acid, 3-hydroxyphenylacetic acid, 3,5-dicaffeoylquinic acid, 3a,6a,7b-trihydroxy-5b-cholanoic acid, apigenin, chlorogenic acid, *o*-coumaric acid, *m*-coumaric acid, gallic acid, isorhamnetin, luteolin, naringin, neochlorogenic acid, phlorizin, protocatechuic acid, pyrogallol, quercetin 4′-glucoside, rutin, secoisolariciresinol, sinapic acid, and *trans*-polydatin from Sigma-Aldrich (Steinhemin, Germany); 3-hydroxybenzoic acid, (−)-epicatechin, ferulic acid, *p*-coumaric acid,

and syringic acid from Fluka (St. Louis, MO, USA); and methyl gallate from Phytolab (Vestenbergsgreuth, Germany). Acetonitrile, formic acid, and methanol were acquired from Sigma-Aldrich (Steinhemin, Germany). All other chemicals were provided by Sigma-Aldrich (Steinhemin, Germany).

2.2. Samples

CS were generously offered by Sortegel (Sortes, Bragança, Portugal) in October 2018. After dehydration (Excalibur Food Dehydrator, CA, USA) at 40 °C for 24 h, CS were milled in an ultra-centrifugal grinder (Retsch model ZM200, Düsseldorf, Germany) to 1 mm of particle size. The powdered samples were stored at room temperature, in sealed flasks placed in the dark.

2.3. In Vitro Simulated Digestion

The simulated gastrointestinal digestion was performed according to the methodology described by Minekus et al. [26], with slight alterations [12,27]. The oral phase was performed by maceration of powdered CS (1 g) with simulated salivary fluid at 1:1 (w/v) ratio containing salivary α-amylase (75 U/mL) at pH 7, followed by incubation in a water bath at 37 °C for 2 min under stirring. Afterward, the gastric phase was performed by adding simulated gastric fluid at a 1:1 (v/v) ratio containing pepsin (2000 U/mL) at pH 3 to the oral bolus and incubation in a water bath at 37 °C for 2 h under stirring. The intestinal phase was then performed by adding simulated intestinal fluid at a 1:1 (v/v) ratio containing pancreatin (100 U/mL) and bile (10 mM) at pH 7 to the gastric digest, followed by incubation in a water bath at 37 °C for 2 h under stirring. In the simulated fluids preparation, KCl, KH_2PO_4, $NaHCO_3$, NaCl, $MgCl_2(H_2O)_6$, and $(NH_4)_2CO_3$ were employed [26]. At the end of each digestion phase, aliquots were collected. After centrifuging at 10,000× g for 10 min, the aliquots were kept at −80 °C until further analysis. This procedure was carried out three times for each digestion phase. The phenolics recovery rate was determined by the following Equation (1):

$$\text{Recovery (\%)} = (PC_{DS}/PC_{FC}) \times 100 \quad (1)$$

where PC_{DS} refers to the phenolic content of the digested sample and PC_{FC} refers to the phenolic content of the undigested sample. The recovery index after all digestion phases corresponds to the bioaccessibility (%).

2.4. Recovery of Phenolic Compounds from Chestnut Shells

The powdered shell samples (1 g) were extracted by maceration with distilled water (20 mL) and heated in a thermostatic water bath (Ovan, BSC127-E model, Barcelona, Spain) at 50 °C for 30 min, according to Rodrigues et al. [22]. After extraction, the sample was filtered through Whatman no. 1 filter paper, centrifuged at 8000 rpm for 5 min (Sigma 3-30KS, Sigma, Osterode am Harz, Germany), lyophilized, and stored at room temperature in the dark for further analysis. The recovery yield was 12.76 ± 1.11% (w/w).

2.5. Total Phenolic Content

The total phenolic content (TPC) was estimated by the Folin–Ciocalteu procedure, according to Pinto et al. [12]. The standard used to plot the calibration curve was gallic acid ($R^2 \geq 0.998$). The results are presented as mg of gallic acid equivalent (GAE) per g of dry weight (DW) (mg GAE/g DW).

2.6. Total Flavonoid Content

The total flavonoid content (TFC) was determined by the aluminum chloride assay, according to Pinto et al. [12]. The standard used to plot the calibration curve was catechin ($R^2 \geq 0.996$). The results are presented as mg of catechin equivalent (CE) per g of DW (mg CE/g DW).

2.7. In Vitro Antioxidant/Antiradical Properties

The in vitro antioxidant/antiradical properties were screened by three different spectrophotometric methods, namely ABTS and DPPH radical scavenging assays and ferric reducing antioxidant power (FRAP), according to Pinto et al. [27]. For ABTS and DPPH radical scavenging assays, ascorbic acid ($R^2 \geq 0.993$) and Trolox ($R^2 \geq 0.994$) were employed as controls, respectively. The results are presented, respectively, as mg of ascorbic acid equivalent (AAE) per g of DW (mg AAE/g DW) and mg of Trolox equivalent (TE) per g of DW (mg TE/g DW). For the FRAP assay, the standard used to plot the calibration curve was ferrous sulphate heptahydrate ($R^2 \geq 0.997$). The results are presented as mg of ferrous sulphate equivalents (FSE) per g of DW (mg FSE/g DW).

2.8. Reactive Oxygen and Nitrogen Species Quenching Assays

The scavenging potential of digested and undigested CS was assessed following the methodologies validated in previous studies [8,12,27] against ROS and RNS, using a Synergy HT Microplate Reader (BioTek Instruments, Winooski, VT, USA). The results of the samples were compared with two positive controls, namely gallic acid and catechin.

2.8.1. Superoxide Anion Radical Quenching Assay

The superoxide anion radical ($O_2^{\bullet -}$) scavenging assay was assessed by the procedure described by Pinto et al. [12]. The non-enzymatic system NADH/PMS/O_2 generates $O_2^{\bullet -}$ that induces the NBT reduction into purple-colored diformazan, with maximum absorbance at 560 nm after 5 min. The results are presented as inhibition, in % or IC_{50} (µg/mL).

2.8.2. Hydrogen Peroxide Quenching Assay

The hydrogen peroxide (H_2O_2) scavenging assay was assessed by the procedure described by Pinto et al. [12], through H_2O_2-mediated ABTS oxidation catalyzed by peroxidase. The absorbance was measured at 405 nm. The results are expressed as inhibition, in % or IC_{50} (µg/mL).

2.8.3. Hydroxyl Radical Quenching Assay

The hydroxyl radical ($HO^{\bullet}$) scavenging assay was performed according to the protocol validated by Valentão et al. [28] through the $HO^{\bullet}$-induced oxidation of 2-deoxyribose. The absorbance was measured at 532 nm. The results are presented as inhibition, in % or IC_{50} (µg/mL).

2.8.4. Hypochlorous Acid Quenching Assay

The hypochlorous acid (HOCl) scavenging assay was performed following the procedure described by Pinto et al. [12], based on the HOCl-mediated DHR oxidation. The fluorescence was measured at 37 °C for 5 min. The excitation and emission wavelengths were set, respectively, at 485 ± 20 and 528 ± 20 nm. The results are expressed as inhibition, in % or IC_{50} (µg/mL).

2.8.5. Peroxyl Radical Quenching Assay

The peroxyl radical ($ROO^{\bullet}$) scavenging capacity was determined by the oxygen radical absorbance capacity (ORAC) assay, through monitoring the $ROO^{\bullet}$-induced fluorescein oxidation at 37 °C for 2 h [12]. The excitation and emission wavelengths were set, respectively, at 485 ± 20 and 528 ± 20 nm. The standard used to plot the calibration curve was Trolox. The results are presented as µg of TE per mg on DW (µg TE/mg DW).

2.8.6. Peroxynitrite Quenching Assay

The peroxynitrite ($ONOO^-$) scavenging assay was performed through $ONOO^-$–mediated DHR oxidation into fluorescent rhodamine following the procedure of Pinto et al. [12]. The assay was conducted in the absence and presence of 25 mM sodium bicarbonate to mimic the physiological CO_2 concentrations. The excitation and emission wavelengths were set,

respectively, at 485 ± 20 and 528 ± 20 nm. The results are expressed as inhibition, in % or IC_{50} (μg/mL).

2.9. Acetylcholinesterase Activity

AChE activity was estimated according to the study of Pinto et al. [19] based on the reaction between 5,5′-dithiobis(2-nitrobenzoic acid) and thiocholine and the amount of colorimetric product formed, with maximum absorbance at 412 nm. A commercial enzymatic kit acquired from Sigma-Aldrich (St. Louis, MO, USA) was used to evaluate AChE activity. The results are presented in inhibition percentage (%).

2.10. Metabolomic Analysis by LC-ESI-LTQ-Orbitrap-MS

The metabolomic analysis was conducted following the method proposed in previous studies [16,18,29], with slight modifications, using a LC-ESI-LTQ-Orbitrap-MS equipment with an Accela chromatograph (Thermo Scientific, Hemel Hempstead, UK), a photodiode array detector, a quaternary pump, and a thermostated autosampler coupled to an LTQ Orbitrap Velos mass spectrometer (Thermo Scientific, Hemel Hempstead, UK) with an ESI source in negative mode. The system control was performed using Xcalibur v3.0 software (ThermoFisher Scientific, Hemel Hempstead, UK). The elution was performed on an Acquity™ UPLC® BEH C18 Column (2.1 × 100 mm, i.d., 1.7 μm particle size, Waters Corporation, Wexford, Ireland) kept at 30 °C. The solvents employed were water (A) and acetonitrile (B) both containing 0.1% formic acid. The solvent gradient (*v*/*v*) of B (t (min), %B) was set as follows: (0, 0); (2, 0); (4, 30); (8, 100); (10, 100); (11, 0); (14, 0). The column temperature, flow rate, and injection volume applied were defined, respectively, at 30 °C, 0.450 mL/min, and 5 μL. The instrumental conditions were defined according to Pinto et al. [16,18]. Samples were analyzed in full scan mode at resolving power of 30,000 and *m*/*z* 600. Data-dependent MS/MS events were acquired at a resolving power of 15,000. Most intense ions were detected in FTMS mode-triggered data-dependent scanning. Ions not sufficiently intense for a data-dependent scan were explored in MS^n mode. Precursors were fragmented by collision-induced dissociation using a C-trap with normalized collision energy (35 V) and activation time of 10 ms. The chromatographic parameters were set as follows: source voltage, 3 kV; sheath gas, 50 a.u. (arbitrary units); auxiliary gas, 20 a.u.; sweep gas, 2 a.u.; and capillary temperature, 375 °C.

Regarding identification, compounds were putatively annotated using MS-finder software and MS-dial software (open source software version 4.24, created by Prof. Masanori Arita team (RIKEN) and Prof. Oliver Fiehn team (UC Davis) supported by the JST/NSF SICORP "Metabolomics for the low carbon society" project, Wakō, Japan, and Sacramento, CA, USA) [30–33] for data treatment, considering the high confidence provided by the fragmentation pattern, isotopic pattern (isotopic spacing and isotopic ratio) followed by exact mass, and retention time alignments. A database set by combining annotations from Phenol-Explorer (http://phenol-explorer.eu/ (accessed on 12 May 2023)) and Food Database (http://foodb.ca/ (accessed on 12 May 2023)) was employed as a reference for putative annotation.

The quantification of the phenolic compounds annotated was performed by plotting the calibration curves with the respective standards. The standard calibration curves were as follows:

2,5-Dihydroxybenzoic acid: y = 338059.71x + 7363.24 (R^2 = 0.9956)
Pyrogallol: y = 470522.52x − 11020.68 (R^2 = 0.9993)
3-Hydroxyphenylacetic acid: y = 44726.40x − 519.58 (R^2 = 0.9965)
Protocatechuic acid: y = 418275.55x − 9848.42 (R^2 = 0.9966)
2,6-Dihydroxybenzoic acid: y = 1514316.77x + 150533.81 (R^2 = 0.9830)
o-coumaric acid: y = 435894.80x + 36088.05 (R^2 = 0.9829)
p-coumaric acid: y = 365335.59x + 22717.00 (R^2 = 0.9912)
m-coumaric acid: y = 477409.77x + 25430.25 (R^2 = 0.9884)
Gallic acid: y = 153094.02x − 4300.35 (R^2 = 0.9993)

Methyl gallate: y = 1648641.24x + 149405.28 (R^2 = 0.9827)
Ferulic acid: y = 103258.90x + 1744.87 (R^2 = 0.9916)
Sinapic acid: y = 137136.36x − 1293.06 (R^2 = 0.9982)
Apigenin: y = 2358556.91x + 244037.75 (R^2 = 0.9876)
Luteolin: y = 2433783.00x + 213408.42 (R^2 = 0.9942)
(−)-Epicatechin: y = 646796.49x + 17432.03 (R^2 = 0.9944)
Isorhamnetin: y = 1392573.78x + 47514.80 (R^2 = 0.9938)
Chorogenic acid: y = 465304.56x + 6580.04 (R^2 = 0.9955)
Neochlorogenic acid: y = 324413.23x + 2119.41 (R^2 = 0.9902)
Secoisolariciresinol: y = 232850.78x + 897.02 (R^2 = 0.9979)
trans-polydatin: y = 191140.72x + 7123.18 (R^2 = 0.9934)
Phlorizin: y = 448036.33x + 3647.70 (R^2 = 0.9985)
Quercetin 4′-glucoside: y = 948205.72x + 32406.83 (R^2 = 0.9877)
3,5-Dicaffeoylquinic acid: y = 354049.31x + 3476.39 (R^2 = 0.9989)
Naringin: y = 557685.80x + 13458.61 (R^2 = 0.9976)
Rutin: y = 522117.78x − 16766.91 (R^2 = 0.9996)

The results are expressed in µg of each phenolic compound equivalent per g on DW (µg/g DW).

2.11. Statistical Analysis

The results are expressed as mean ± standard deviation resulting from at least three independent experiments. One-way ANOVA and Tukey's HSD test were conducted for the statistical analysis using IBM SPSS Statistics 24.0 software (Chicago, IL, USA). The results with statistical significance were denoted for $p < 0.05$.

3. Results

3.1. Effect of In Vitro Digestion in the Phenolic and Flavonoid Contents

The impacts of the in vitro simulated digestion on the TPC and TFC of CS are presented in Figure 1A and B, respectively.

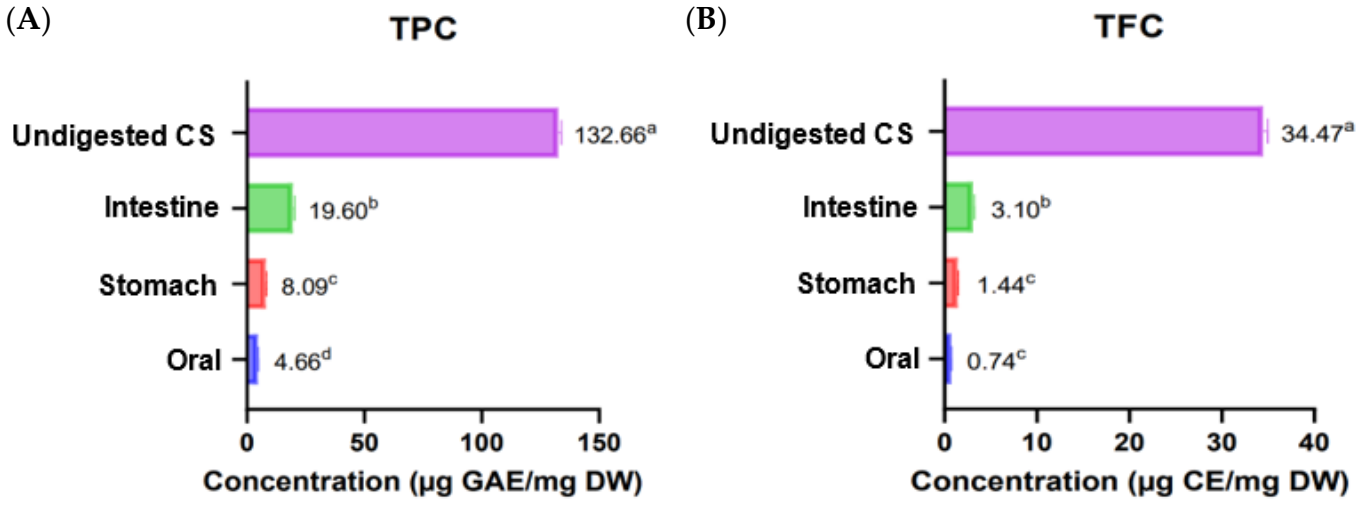

Figure 1. TPC (**A**) and TFC (**B**) of chestnut shells before and after in vitro digestion. Different letters at superscript (a, b, c, and d) indicate significant differences ($p < 0.05$) between samples.

As shown in Figure 1A, the TPC was raised during the in vitro digestion in the following order: oral < gastric < intestinal digests, although the undigested CS showed the highest result (132.66 µg GAE/mg DW). From oral to gastric digestion, the TPC increased by 74%, while a 2.4-fold increase was achieved from gastric to intestinal digestion, proving that the intestinal enzymes and the pH conditions (i.e., pancreatin, bile salts, and neutral pH) enabled a higher release of phenolics from CS matrix to the digestion medium. In

addition, the TPC decreased 6.8-fold after digestion when compared to the undigested fraction. Significantly different results ($p < 0.05$) were obtained from the three digests and the undigested CS.

The TFC was also determined for all digestion phases and the undigested CS, revealing lower concentrations for digests when compared to the undigested sample (34.47 µg CE/mg DW). A significant increase of flavonoid concentrations during the digestion was attested, following this order: 0.74, 1.44, and 3.10 µg CE/mg DW, respectively, for oral, gastric, and intestinal digests. Noteworthily, a 2-fold increase in TFC was observed from oral to gastric digestion, while the TFC increased 2.2-fold from gastric to intestinal digestion. Conversely, the TFC decreased 11-fold after digestion, when compared to the undigested fraction. Additionally, the undigested CS and its intestinal digest revealed significantly different TFC values ($p < 0.05$), whereas non-significant differences ($p > 0.05$) were observed between oral and gastric digests. A similar conclusion to that for TPC may be hypothesized, suggesting that flavonoids are more easily recovered under an intestinal environment, reinforcing the positive influence of pancreatin and neutral pH in the release of flavonoids from the CS matrix.

In summary, changes in TPC and TFC have been noted upon in vitro gastrointestinal digestion, highlighting that phenolic and flavonoid concentrations rely on the digestion phase, principally owing to the instability of phenolics exposed to digestive enzymes and pH alterations that endorse their degradation and biotransformation into simpler molecules. In fact, previous studies have already proven similar outcomes with higher phenolic and flavonoid concentrations determined after intestinal digestion when compared to the oral digestion, also attesting the highest concentrations of these molecules in the undigested samples [34–37]. This may be not only due to the action of digestive enzymes and pH changes that enable a gradual release of phenolics and flavonoids during digestion (leading to an increase of TPC and TFC during in vitro digestion) but also owing to the solubility of these molecules in the digestion media (presenting a higher solubility in the intestinal digestion medium and low solubility in the oral medium). Recent advances have also demonstrated an increase in TPC and TFC during gastrointestinal digestion of different fruit by-products, with improved phenolic recovery after the intestinal phase and the highest concentrations determined in the undigested samples [34–36].

Notably, the TPC and TFC of digested fractions may be underestimated since the methodologies employed only determine the soluble phenolic compounds, neglecting the presence of condensed phenolic compounds, proanthocyanidins, and other insoluble polymeric phenolic compounds that are not recovered in the aqueous digestion media. Additionally, soluble and insoluble fiber abundant in CS may interfere with these spectrophotometric assays for the quantification of TPC and TFC.

3.2. Bioaccessibility

The recovery rates of phenolic compounds from CS after each digestion phase are presented in Figure 2.

As expected, the phenolic recovery improved during in vitro digestion. The lowest recovery rate was achieved after the oral phase (3.52%) and increased significantly ($p < 0.05$) after gastric (6.79%) and intestinal (16.47%) phases. These findings corroborate the TPC results and reinforced the hypothesis that intestinal enzymes (e.g., pancreatin and bile salts) and neutral pH exerted a major influence on the recovery of phenolic compounds from CS when compared to the gastric and oral environments.

The low phenolic recovery may be explained by possible interference of phenolic compounds with other CS components (such as oligosaccharides, lignins, and fatty acids) that may hinder the release of phenolics upon digestion [36]. In addition, the low phenolic recovery may be associated with the solubility and polarity of these compounds in the digestive media [27].

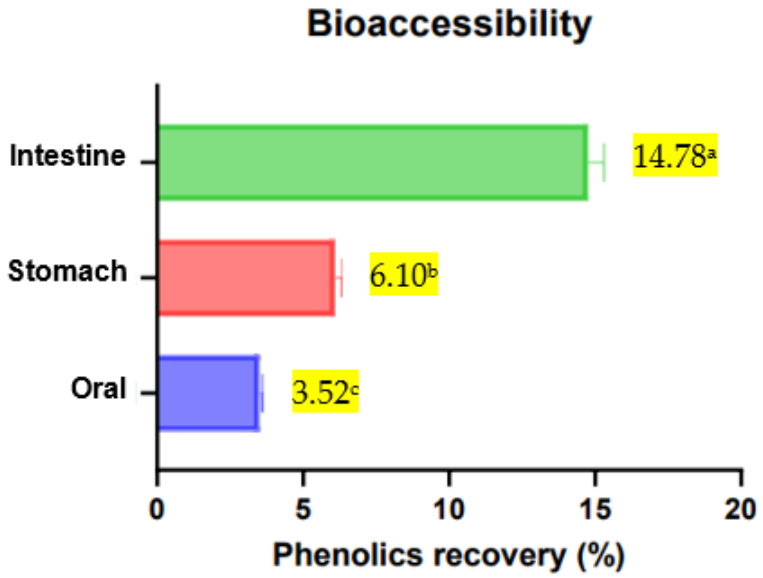

Figure 2. Recovery rates of phenolic compounds after each digestion phase. Different letters at superscript (a, b, and c) indicate significant differences ($p < 0.05$) between samples.

The bioaccessibility of phenolic compounds was estimated at almost 20%, representing the recovery rate after all digestion phases. These results outline a slow-release profile of bioactive molecules from CS, considering that bioaccessibility corresponds to the concentration of phenolics released from the matrix during the digestion process to become available for absorption. The main concluding remark of these results is that phenolic compounds entrapped in CS probably become more bioaccessible during gastrointestinal digestion since their recovery rates increased from oral to intestinal phases and achieved the maximum value of 15% after intestinal digestion. Additionally, identical findings were reported in preceding studies on fresh blueberry (<15.1%), and mulberry leaves (<15%) [36,38].

3.3. Effect of In Vitro Digestion on the Antioxidant/Antiradical Properties

The impact of in vitro simulated digestion on the antioxidant/antiradical properties of CS was also investigated and the results are presented in Figure 3A–C, respectively, for ABTS, DPPH, and FRAP assays.

According to Figure 3A, the antiradical activity evaluated by scavenging of ABTS radicals improved significantly ($p < 0.05$) during digestion. The best ABTS scavenger was the undigested CS (112.03 µg AAE/mg DW), followed by its intestinal digest (13.35 µg AAE/mg DW). The gastric digest showed a 2.6-fold lower antiradical response when compared to the intestinal digest, while the oral digest had a negligible effect on ABTS scavenging. Only undigested CS and its intestinal fraction disclosed significantly different results ($p < 0.05$), while non-significant differences ($p > 0.05$) were noted between oral and gastric digests. An identical pattern was noted for the DPPH radical scavenging assay (Figure 3B), showing the highest result for the undigested extract (169.25 µg AAE/mg DW) and a significant increment in the DPPH scavenging response during the digestion. A 60% increase was achieved from oral to gastric digestion, while the antiradical activity increased by 54% from gastric to intestinal digestion. All digested and undigested fractions attained significant differences ($p < 0.05$).

Considering Figure 3C, the antioxidant activity evaluated by FRAP assay reached the highest result for the undigested CS (247.46 µg AAE/mg DW), followed by its intestinal digest (44.82 µg AAE/mg DW). The antioxidant response was enhanced 49% from oral to gastric digestion, and 76% from gastric to intestinal digestion. Only undigested CS and its intestinal fraction achieved significantly different results ($p < 0.05$), whereas non-significant differences ($p > 0.05$) were noted between oral and gastric digests. These findings propose that antioxidant compounds are more efficiently recovered under intestinal environment

(probably due to the action of pancreatin and neutral pH), or that the molecules retained exhibit better antioxidant effects. This explanation was also recently provided for coffee beans [37].

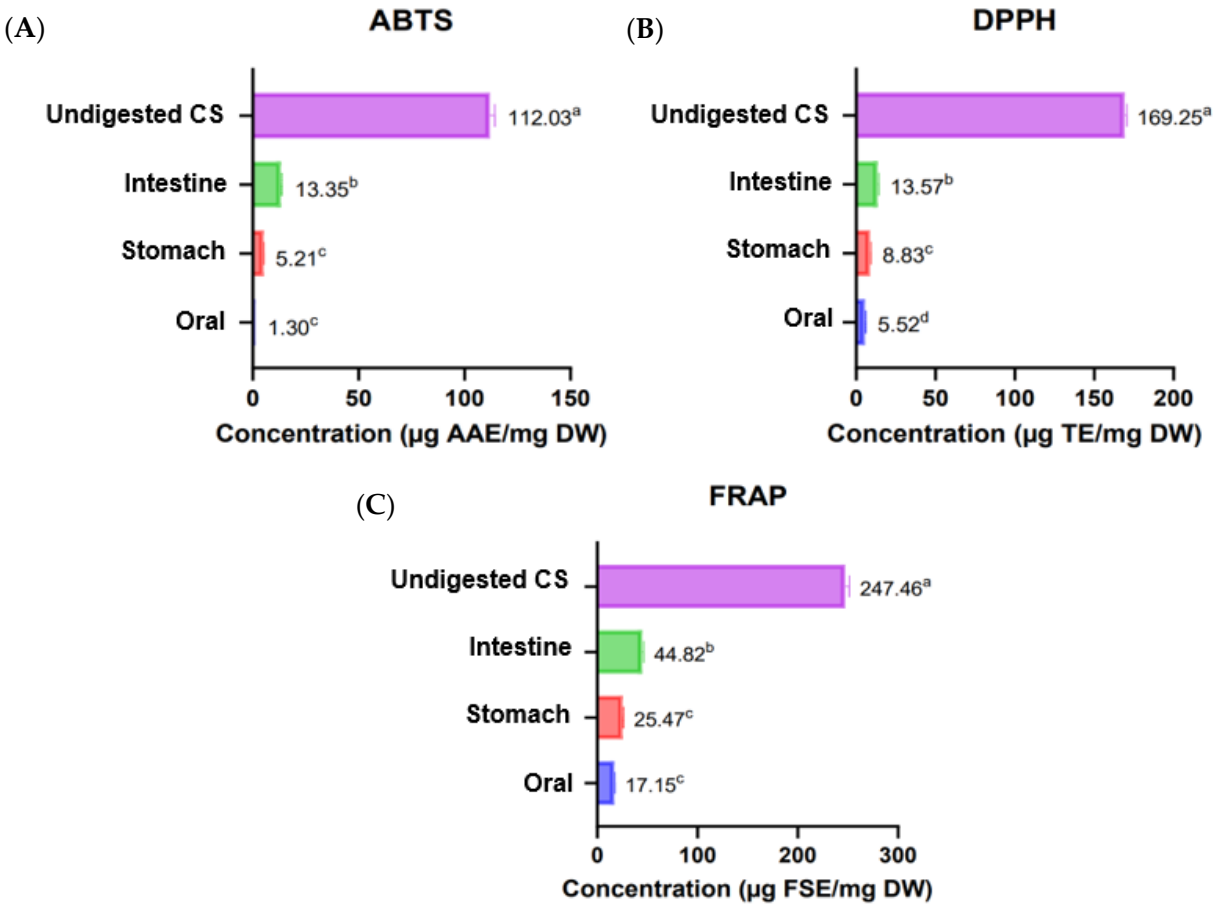

Figure 3. Antioxidant/antiradical properties of chestnut shells before and after in vitro digestion evaluated by ABTS (**A**), DPPH (**B**), and FRAP (**C**) assays. Different letters at superscript (a, b, and c, and d) indicate significant differences ($p < 0.05$) between samples.

In summary, the antioxidant/antiradical properties improved in the following manner: oral < gastric < intestinal digests < undigested CS, highlighting better antioxidant responses for the undigested fraction, along with an upgrading during in vitro digestion. These results are in line with previous reports on the digestibility of antioxidants from fruits and by-products [34,36,37]. The antioxidant/antiradical results are in close accordance with TPC, showing the prominent role of phenolic compounds in the antioxidant/antiradical responses of undigested and digested CS, which may be associated with the increase of phenolic hydroxyl groups released from monomers or aglycones under intestinal environment, offering better antioxidant properties [37]. The latest studies have also demonstrated better TPC, antioxidant/antiradical activities, and bioaccessibility of phenolic compounds from food extracts upon gastrointestinal digestion [34–36]. For instance, the present data agree with a preceding report on Chinese chestnut shells (up to 100 and 90 mg AAE/100 mL, respectively, for gastric and intestinal phases) [25].

3.4. Scavenging Activity against Reactive Oxygen and Nitrogen Species upon In Vitro Digestion

Table 1 presents the results on the scavenging potential of undigested and digested CS against pro-oxidant reactive species produced endogenously in the biological tissues.

Table 1. Scavenging potential of undigested and digested CS against ROS and RNS.

	Reactive Oxygen Species									Reactive Nitrogen Species			
	$O_2^{\bullet-}$		H_2O_2		$HO^{\bullet}$		HOCl		$ROO^{\bullet}$	$ONOO^-$ with $NaHCO_3$		$ONOO^-$ without $NaHCO_3$	
	Inhibition (%)	IC_{50} (μg/mL)	Inhibition (%)	IC_{50} (μg/mL)	Inhibition (%)	IC_{50} (μg/mL)	Inhibition (%)	IC_{50} (μg/mL)	μmol TE/ 100 mg DW	Inhibition (%)	IC_{50} (μg/mL)	Inhibition (%)	IC_{50} (μg/mL)
Oral	14.13 ± 0.90^{3}	–	9.70 ± 1.27^{1}	–	55.23 ± 0.63^{1}	–	23.37 ± 1.49^{3}	–	2.85 ± 0.04^{c}	28.55 ± 0.28^{3}	–	24.46 ± 0.77^{3}	–
Stomach	28.04 ± 0.43^{2}	–	25.84 ± 0.32^{2}	–	64.08 ± 0.41^{2}	–	36.75 ± 0.96^{2}	–	9.76 ± 0.34^{c}	45.11 ± 0.48^{2}	–	40.78 ± 0.17^{2}	–
Intestine	43.01 ± 1.38^{1}	–	39.14 ± 0.95^{3}	–	68.06 ± 0.10^{3}	–	59.85 ± 0.20^{1}	–	19.40 ± 0.49^{c}	77.38 ± 2.28^{1}	–	70.13 ± 0.79^{1}	–
Undigested CS	–	94.89 ± 4.18^{a}	–	77.66 ± 4.45^{b}	–	415.29 ± 2.40^{a}	–	25.45 ± 0.31^{a}	30.01 ± 0.92^{c}	–	14.04 ± 0.33^{a}	–	17.47 ± 0.31^{a}
Positive controls													
Catechin	–	50.97 ± 0.51^{b}	–	20.78 ± 0.75^{c}	–	1.45 ± 0.04^{c}	–	0.38 ± 0.01^{c}	180.96 ± 12.01^{a}	–	0.24 ± 0.01^{b}	–	0.24 ± 0.01^{b}
Gallic acid	–	12.85 ± 0.18^{c}	–	106.03 ± 0.93^{a}	–	63.92 ± 2.75^{b}	–	1.79 ± 0.01^{b}	107.58 ± 10.39^{b}	–	0.27 ± 0.02^{b}	–	0.27 ± 0.01^{b}

IC_{50} refers to the in vitro concentration needed to scavenge 50% of the pro-oxidant species in a tested medium (mean ± standard error of the mean). Different letters (a, b, and c) denote significant differences ($p < 0.05$) between IC_{50} results of positive controls and undigested sample. Different numbers (1, 2, and 3) denote significant differences ($p < 0.05$) between inhibition percentages of digests. Inhibition percentages were determined directly in the aliquots collected from each digestion phase (oral, gastric, and intestinal phases) after the time required.

The counteracting efficiency improved during the digestion following this order: oral < gastric < intestinal digests, which was corroborated by the increasing inhibition percentages attained for all ROS and RNS studied. However, the highest quenching potential was attained for the undigested CS (making it possible to determine the IC_{50}). As expected, the scavenging proficiency on HOCl and $ONOO^-$ showed the best outcomes.

The $O_2^{\bullet -}$ scavenging ability of undigested CS (IC_{50} = 94.89 μg/mL) was the highest within the samples. Among digests, the intestinal fraction (43.01% inhibition) unveiled the best $O_2^{\bullet -}$ quenching potential associated with the highest inhibition percentage, while gastric and oral digests disclosed lower quenching capacity by inhibiting, respectively, 28.04% and 14.13% of $O_2^{\bullet -}$ production. Gallic acid (IC_{50} = 12.85 μg/mL) and catechin (IC_{50} = 50.97 μg/mL) displayed better capacity to scavenge $O_2^{\bullet -}$ when compared to undigested CS due to the lowest IC_{50} values (considering that lower IC_{50} values indicate higher scavenging capacity). All digests and undigested CS showed significantly different scavenging responses ($p < 0.05$).

Considering the H_2O_2 quenching assay, the best scavenger among the samples was the undigested extract (IC_{50} = 77.66 μg/mL). Among digests, the intestinal fraction showed the highest H_2O_2 scavenging ability associated with the highest inhibition percentage (39.14% inhibition), while gastric and oral digests achieved lower quenching potential with 25.84% and 9.70% H_2O_2 inhibition, respectively. Furthermore, the undigested CS disclosed a significantly ($p < 0.05$) lower IC_{50} value when compared to gallic acid (IC_{50} = 106.03 μg/mL), indicating that undigested CS exhibited a higher H_2O_2 scavenging ability than gallic acid. Otherwise, the lowest IC_{50} was attained for catechin (IC_{50} = 20.78 μg/mL), emphasizing a higher H_2O_2 quenching efficiency when compared to undigested CS. Significant differences ($p < 0.05$) were determined between all samples.

Regarding the $HO^{\bullet}$ counteracting assay, intestinal and gastric digests (68.06% and 64.08% inhibition, respectively) quenched more efficiently this species than the oral digest (55.23% inhibition) owing to their higher inhibition percentages. Additionally, the undigested CS showed a better $HO^{\bullet}$ scavenging capacity than its digests, making it possible to determine the IC_{50} (415.29 μg/mL), which was 6.5-fold higher than the IC_{50} value of gallic acid (63.92 μg/mL), indicating that the undigested CS had a lower $HO^{\bullet}$ quenching potential when compared to gallic acid. Additionally, catechin was the best $HO^{\bullet}$ scavenger associated with the lowest IC_{50} obtained (1.45 μg/mL). Significantly different results ($p < 0.05$) were determined between all samples and the positive controls.

Concerning the HOCl assay, the highest quenching response among the samples was achieved for the undigested extract (IC_{50} = 25.45 μg/mL). The intestinal digest reached the highest inhibition percentage (59.85%) among digests, denoting a better ability to scavenge HOCl, followed by gastric and, finally, oral digests (36.75% and 23.37% HOCl inhibition, respectively). Catechin (IC_{50} = 0.38 μg/mL) was the best quencher for this species associated with the lowest IC_{50} determined, followed by gallic acid (IC_{50} = 1.79 μg/mL). All digests and undigested sample displayed significantly different results ($p < 0.05$).

ORAC assay was used to evaluate the $ROO^{\bullet}$ counteracting power and, subsequently, estimate the protective effects against lipid peroxidation injuries [8]. The $ROO^{\bullet}$ scavenging potential of undigested CS and its oral, gastric, and intestinal digests was identical (30.01, 2.85, 9.76, and 19.40 μmol TE/100 mg DW, respectively), without significant differences ($p < 0.05$). Conversely, significantly higher results ($p < 0.05$) were obtained for catechin and gallic acid (180.96 and 107.58 μmol TE/100 mg DW, respectively).

Regarding the $ONOO^-$ scavenging assay, $NaHCO_3$ was used to simulate the physiological bicarbonate concentrations present in the human body (≈25 mM) [8]. The procedure was performed in the presence and absence of $NaHCO_3$, considering that certain phenolic compounds prompted a more prominent reduction on the $ONOO^-$ counteracting ability in a tested medium containing $NaHCO_3$ (e.g., caffeic, *p*-coumaric, ferulic, and gallic acids) unlike others (e.g., catechin derivatives) [39]. The $ONOO^-$ scavenging proficiency was enhanced in the following order: oral < gastric < intestinal digests < undigested extract. The best outcomes were achieved in the presence of $NaHCO_3$ (28.55%, 45.11%, and 77.38%

inhibition and IC_{50} of 14.04 μg/mL, respectively, for oral, gastric, and intestinal digests and undigested CS) associated with the highest inhibition percentages attained for the digests and lowest IC_{50} determined for the undigested CS, when compared to its absence (24.46%, 40.78%, 70.13% inhibition, and IC_{50} of 17.47 μg/mL). Otherwise, the scavenging capacities of catechin and gallic acid were similar ($p > 0.05$) in the tested media with or without $NaHCO_3$. Additionally, significantly different results ($p < 0.05$) were disclosed for digested and undigested samples.

The present results were slightly lower than the ones reported for CS extracts prepared by eco-friendly techniques [6,7,9,15,19], emphasizing the importance of selecting a suitable extraction technique to isolate phenolic compounds. A recent paper demonstrated a higher bioaccessibility and bioactivity of a phenolic-rich extract from CS prepared by subcritical water extraction (SWE) after in vitro digestion [27]. Hence, the extraction technique may be a good alternative to improve the bioaccessibility of phenolic compounds when compared to the use of food by-products by themselves. Moreover, the drop on ROS/RNS scavenging proficiency after in vitro digestion was also corroborated by the previous TPC, TFC, and antioxidant/antiradical results presented [12,36,40]. In fact, the results on ROS/RNS scavenging potential were concordant with those for TPC and TFC (Figure 1). A similar tendency was observed, with increasing TPC, TFC, and ROS/RNS scavenging efficiency in the following order: oral digest < gastric digest < intestinal digest < undigested CS. Among samples, the undigested extract attained the highest TPC and TFC and low IC_{50} values determined for ROS/RNS assays, which were associated with the highest ROS/RNS scavenging potential, proposing the strong contribution of phenolic compounds and flavonoids to the highest radical scavenging proficiency of undigested CS. Among digests, the intestinal fraction achieved the highest TPC and TFC, along with the highest inhibition percentages for ROS/RNS assays, emphasizing the role of phenolic compounds and flavonoids recovered during intestinal digestion in the radical scavenging properties when compared to gastric and oral digests. Conversely, the oral digest had the lowest TPC and TFC, which corroborated the lowest radical scavenging potential associated with the lowest inhibition percentages for all ROS and RNS studied.

The findings on ROS/RNS scavenging efficiency pointed out the promising role of phenolic composition in the bioactivity of digested and undigested CS (particularly hydroxybenzoic acids, hydroxycinnamic acids, flavanols, flavonols, and lignans), whose counteracting responses have been attested in recent research [27,41,42].

3.5. Inhibition of Acetylcholinesterase Activity upon In Vitro Digestion

Aside from antioxidant properties, agri-food by-products rich in phenolic compounds deliver protective effects against neurodegeneration by inhibition of AChE and butyrylcholinesterase activities, exerting neuroprotective properties [43]. Most studies have investigated the neuroprotective properties by inhibition of AChE activity directly in phenolic-rich extracts from agri-food by-products [8,19,40]. Nevertheless, this approach might potentially overestimate their anti-cholinergic effects by neglecting the impact of in vitro digestion on the bioaccessibility and bioactivity of phenolic compounds. In this study, the AChE activity was assessed in the undigested CS and after each digestion phase to ascertain whether this activity improved during the simulated gastrointestinal digestion, following the tendency of TPC, antioxidant, and antiradical properties, and showing potential correlations between AChE activity and phenolic composition. Noteworthily, the phenolic compounds only exerted their neuroprotective effects by inhibition of AChE activity after being digested and absorbed, mainly in the intestinal lumen. Therefore, this study provides a preliminary assessment of the neuroprotective properties mediated by phenolic compounds from CS throughout the digestion process. Figure 4 depicts the results on the inhibition of AChE activity of CS upon in vitro digestion.

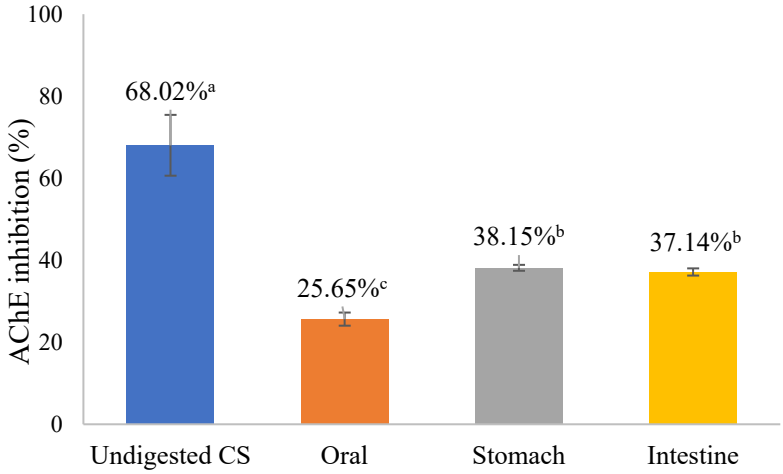

Figure 4. Inhibition of AChE activity before and after in vitro digestion of CS. Different letters (a, b, and c) denote significant differences ($p < 0.05$) between samples.

The highest AChE inhibition was achieved for the undigested CS (68.02% inhibition), while a 2.7-fold reduction was disclosed after oral digestion (25.65% inhibition) and 1.8-fold reduction after gastric and intestinal digestion (38.15% and 37.14% inhibition, respectively). Significant differences ($p < 0.05$) were attained for undigested CS and the oral phase, while the results of gastric and intestinal digests were not significantly different ($p > 0.05$). Even though the undigested CS revealed the best outcomes, an improvement of anti-cholinergic activity was shown during in vitro digestion. These findings are in line with the TPC results, indicating that CS phenolic compounds were slowly released during digestion and, consequently, led to the neuroprotective properties evidenced. Recently, the influence of in vitro digestion was also studied in a phenolic-rich CS extract prepared by SWE regarding anti-cholinergic effects, revealing better results (27.13%, 45.35%, 45.19%, and 74.47% AChE inhibition, respectively, for oral, gastric, and intestinal digests and undigested CS), which highlights the hypothesis proposed that the implementation of an appropriate extraction technique may be helpful to improve the bioaccessibility of CS phenolic compounds and, consequently, the bioactivity [27].

The neuroprotective effects observed are probably attributed to the phenolic composition of the undigested and digested CS. Noteworthily, the main phenolic compounds identified, namely hydroxybenzoic acids, hydroxycinnamic acids, flavanols, and lignans, have been indicated as effective bioactive molecules in the prevention and co-therapy of neurodegenerative diseases, owing to their promising inhibitory effects on AChE activity [40,44,45]. This explanation is also sustained by the strong positive correlations of anti-AChE activity with TPC ($r^2 = 0.94$) and TFC ($r^2 = 0.94$).

3.6. Metabolomic Profiling of Chestnut Shells by LC-ESI-LTQ-Orbitrap-MS upon In Vitro Digestion

The untargeted metabolomic profiling by LC-ESI-LTQ-Orbitrap-MS was studied to outline the changes in phytochemical composition (with a major focus on phenolic compounds) influenced by the digestion process [25]. Supplementary Table S1 provides a detailed list containing each putatively annotated compound detected in undigested CS and its digested fractions from oral, gastric, and intestinal phases, pointing out the fate of different classes of compounds during the in vitro digestion. A total of 76 compounds were putatively annotated, with a higher abundance of polyphenols (35 annotated compounds) representing almost 50% of the total number of compounds identified, followed by lipids (17 compounds representing 22%), phenylpropanoids and polyketides (7 compounds representing 9%), organic acids and derivatives (5 compounds representing 7%), carbohydrates (4 compounds representing 5%), nucleosides, nucleotides, and analogues (4 compounds

representing 5%), amino acids and analogues (3 compounds representing 4%), and alcohols or polyols (1 compound representing 1%).

The quantitative analysis of the phenolic compounds was performed using their respective standards or the most similar compounds (when the standard was not available) to examine the impact of the in vitro simulated digestion (particularly oral, gastric, and intestinal phases) on the phytochemical composition of CS by comparison with its undigested fraction. Table 2 summarizes the results.

Table 2. Quantification of phenolic compounds in undigested and digested CS by LC-ESI-LTQ-Orbitrap-MS.

Phenolic Metabolites	Concentration (μg/g DW)			
	In Vitro Digestion			Undigested CS
	Oral	Stomach	Intestine	
3-Hydroxybenzoic acid	3.05 ± 1.86 ^{b}	6.74 ± 4.63 ^{b}	n.q.	188.78 ± 14.80 ^{a}
3-Hydroxyphenylacetic acid	1.34 ± 0.27 ^{c}	2.61 ± 1.65 ^{c}	6.72 ± 4.36 ^{b}	557.74 ± 41.63 ^{a}
2,5-Dihydroxybenzoic acid	n.d.	n.d.	n.d.	24.40 ± 2.72
p-Coumaric acid	tr	tr	tr	10.71 ± 0.19
Epicatechin	n.d.	n.d.	n.d.	95.75 ± 9.52
Ferulic acid	tr	tr	n.d.	49.71 ± 7.00
Gallic acid	48.36 ± 3.08 ^{c}	84.90 ± 26.25 ^{b}	n.d.	3978.74 ± 290.95 ^{a}
Isorhamnetin	n.d.	n.d.	n.d.	5.29 ± 0.21
Methyl gallate	n.q.	n.d.	n.d.	3.63 ± 1.58
Neochlorogenic acid	n.d.	n.d.	n.d.	2.47 ± 0.73
Phlorizin	n.d.	n.d.	n.d.	3.96 ± 1.14
Protocatechuic acid	2.06 ± 0.12 ^{b}	2.90 ± 1.65 ^{b}	1.17 ± 0.17 ^{c}	106.80 ± 6.92 ^{a}
Pyrogallol	4.40 ± 0.31 ^{b}	7.13 ± 4.48 ^{b}	tr	407.26 ± 61.66 ^{a}
Quercetin 4′-glucoside	n.d.	n.d.	n.d.	7.12 ± 1.23
Secoisolariciresinol	0.02 ± 0.01 ^{b}	n.d.	n.d.	7.49 ± 1.08 ^{a}
Sinapic acid	0.11 ± 0.01 ^{b}	n.d.	n.d.	25.62 ± 3.92 ^{a}
Syringic acid	13.84 ± 6.67 ^{b}	n.d.	n.d.	305.60 ± 37.21 ^{a}

n.d., not detected. tr, trace concentrations. Different letters (a, b, and c) denote significant differences ($p < 0.05$) between samples.

As shown in Table 2, the phenolic concentration was highest in the undigested CS and decreased significantly upon digestion. A total of 17 compounds were quantified in the undigested CS. Gallic acid was the most abundant phenolic compound quantified, representing 68.82% of the total concentration, followed by 3-hydroxyphenylacetic acid (9.65%), pyrogallol (7.04%), syringic acid (5.29%), 3-hydroxybenzoic acid (3.26%), and protocatechuic acid (1.85%). There were considerable amounts of epicatechin (1.66%), ferulic acid (0.86%), sinapic acid (0.44%), 2,5-dihydrozybenzoic acid (0.42%), and *p*-coumaric acid (0.19%). In addition, secoisolariciresinol (0.13%), quercetin 4′-glucoside (0.12%), isorhamnetin (0.09%), phlorizin (0.07%), methyl gallate (0.06%), and neochlorogenic acid (0.04%) were quantified in lower concentrations.

Regarding the digested samples, eight compounds were quantified after oral digestion, while five compounds were quantified after gastric phase. Only two compounds were quantified after intestinal digestion. Considering the oral digest, gallic acid (66.08%) was the major phenolic compound, followed by syringic acid (18.91%), pyrogallol (6.01%), 3-hydroxybenzoic acid (4.17%), protocatechuic acid (2.81%), and 3-hydroxyphenylacetic acid (1.83%), while residual concentrations of sinapic acid (0.15%) and secoisolariciresinol (0.03%) were determined. In the gastric digest, gallic acid was also quantified in highest concentration (81.42%), along with substantial amounts of pyrogallol (6.84%), 3-hydroxybenzoic acid (6.46%), protocatechuic acid (2.78%), and 3-hydroxyphenylacetic acid (2.50%). Concerning the intestinal digest, 3-hydroxyphenylacetic acid was the most abundant phenolic compound (85.17%), followed by protocatechuic acid (14.83%). In a previous report, Tu et al. [25] detected epicatechin, isorhamnetin-7-*O*-glucoside, and methyl gallate in Chi-

nese chestnut inner and outer shells after in vitro simulated digestion. However, the authors only reported a relative content of phenolic compounds, lacking a detailed quantification.

Most of the phenolic compounds quantified in the undigested CS were not detected in its digested fractions. A possible explanation is that the phenolic compounds present in chestnut shells (mainly lignans, flavonoids, and hydroxycinnamic acids) were degraded during digestion owing to the action of enzymes and pH changes. Furthermore, some phenolic compounds may also become insoluble in the digestion media (such as lignans and some flavonoids), as described in previous studies [12,24,27].

In agreement with the results of spectrophotometric assays, the in vitro simulated digestion induced a significant impact on the phytochemical profile of CS, reinforcing the role of digestive enzymes and pH changes in the biotransformation of phytochemicals, particularly phenolic compounds, into metabolites [12]. The discrepancies between the results of chromatographic analysis and the spectrophotometric assays may be explained by the limitations of spectrophotometric methods, including relatively low sensitivity and selectivity, difficulties in detecting low concentrations of analytes, and interference with other constituents of food matrices (e.g., reducing sugars, lipids, and vitamin C) that are absorbed at the same wavelength as phenolic compounds [46].

3.7. Screening of Potential Correlations between Phenolic Composition and Bioactivity upon In Vitro Digestion

The correlations between phenolic composition and bioactivity of CS are outlined in the heatmap diagram presented in Figure 5.

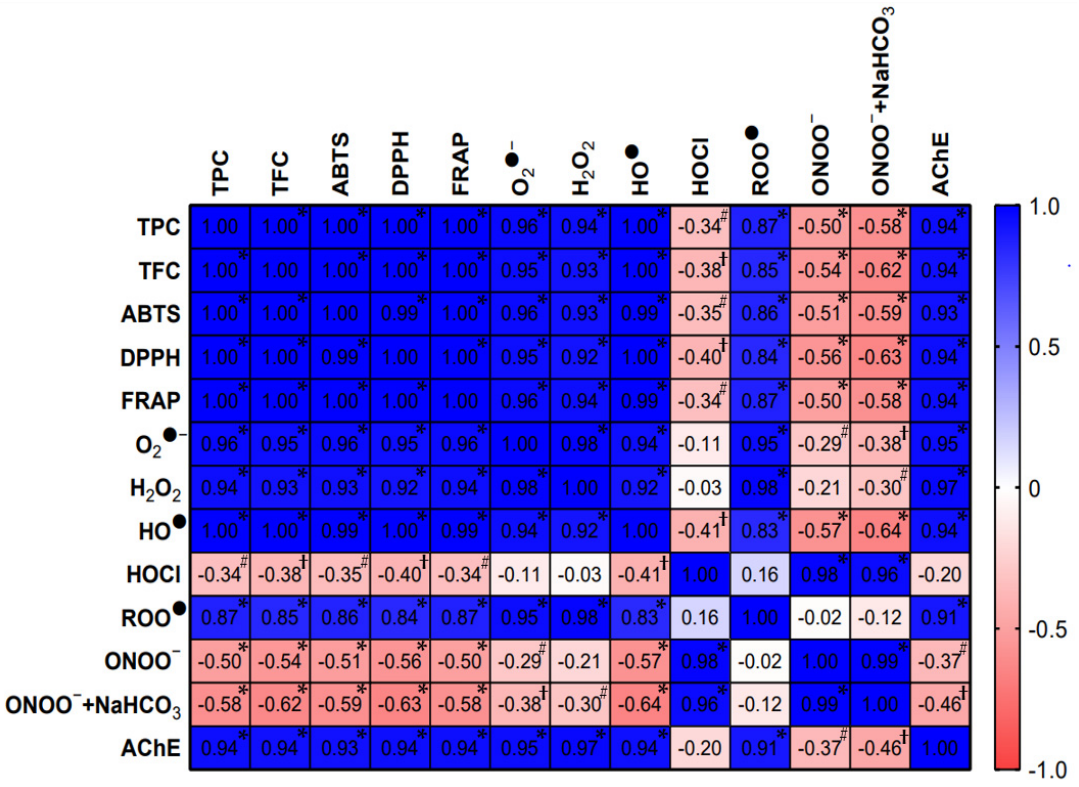

Figure 5. Heatmap correlations of in vitro bioactivity and phenolic composition of digested and undigested CS. * indicates $p < 0.001$; † indicates $p < 0.01$; # indicates $p < 0.05$.

As shown in Figure 5, TPC and TFC were positively correlated with ABTS, DPPH, FRAP, $O_2^{\bullet-}$, H_2O_2, $HO^{\bullet}$, $ROO^{\bullet}$, and AChE based on r^2 values above 0.94 and 0.93, respectively, emphasizing the strong contribution of phenolic compounds to the antioxidant/antiradical activities, scavenging potential against ROS and RNS, and neuroprotective properties of CS upon in vitro digestion. All these correlations were revealed to be

significant with $p < 0.001$. The correlation between TPC and TFC was also denoted as strong ($r^2 = 1.00$) and significant ($p < 0.001$). Conversely, mild to weak negative correlations were determined between TPC and the scavenging assays on HOCl ($p < 0.05$) and $ONOO^-$ in the presence and absence of $NaHCO_3$ ($p < 0.001$), based on r^2 values below -0.58, suggesting that these antiradical responses were probably not prompted by phenolic compounds. In addition, identical correlations were found between TFC and these scavenging assays, with r^2 values below -0.62, underlining that flavonoids poorly contribute to the scavenging efficiency of HOCl and $ONOO^-$. These correlations were also significant with $p < 0.01$ for HOCl and $p < 0.001$ for both $ONOO^-$ assays. Therefore, the remarkable contribution of phenolic compounds abundantly produced in CS to its bioactivity was highlighted by the strong positive correlations determined and corroborated by previous studies with identical outcomes on other food by-products [24,25,37,38,40,41].

The in vitro antioxidant activity evaluated by ABTS, DPPH, and FRAP assays, as well as scavenging efficiency against $O_2^{\bullet -}$, H_2O_2, $HO^{\bullet}$, $ROO^{\bullet}$, and anti-cholinergic activity by AChE inhibition, showed significant ($p < 0.001$) positive correlations with r^2 values above 0.84. Otherwise, ABTS, DPPH, and FRAP responses exerted significant ($p < 0.05$) negative correlations with HOCl and $ONOO^-$ scavenging assays based on r^2 values below -0.63.

The neuroprotective effects of AChE inhibition were positively correlated with TPC, TFC, antioxidant properties evaluated by ABTS, DPPH, and FRAP assays, and scavenging potential against $O_2^{\bullet -}$, H_2O_2, $HO^{\bullet}$, and $ROO^{\bullet}$ based on significant ($p < 0.001$) r^2 values above 0.91. Nonetheless, the anti-cholinergic activity of AChE inhibition negatively correlated with $ONOO^-$ scavenging assay in the presence and absence of $NaHCO_3$ (r^2 values of -0.46 and -0.37, respectively, with $p < 0.01$ and $p < 0.05$), while no significant ($p > 0.05$) correlation was attained with HOCl. These results highlight the strong correlations between antioxidant, ROS and RNS scavenging efficiency, and neuroprotective properties of CS.

4. Conclusions

As concluding remarks, the present findings proved that CS are a prominent source of phytochemicals, namely phenolic compounds that potentially exert biological activities, even after in vitro digestion. Phenolic concentrations and their bioaccessibility decreased after CS digestion, when compared to the undigested fraction, but increased from oral to intestinal digestion. Additionally, the CS still exerted antioxidant/antiradical properties, reactive oxygen and nitrogen species scavenging efficiency, and neuroprotective effects upon in vitro digestion, corroborating the TPC and TFC results, along with their positive correlations outlined by heatmap analysis. The metabolic profiling of targeted phenolic compounds seemed to greatly influence the antioxidant/antiradical scavenging, and neuroprotective properties of CS upon in vitro digestion. The CS were mainly composed of gallic acid (68.82%), 3-hydroxyphenylacetic acid (9.65%), pyrogallol (7.04%), and syringic acid (5.29%). The metabolomic profiling by LC-ESI-LTQ-Orbitrap-MS showed changes to the phytochemical composition during the simulated digestion, particularly of phenolic compounds (46% of the total number of compounds annotated), lipids (22%), phenylpropanoids (9%), organic acids (7%), carbohydrates (5%), nucleosides (5%), amino acids (4%), and alcohols (1%). Nevertheless, the phenolic compounds were relatively unstable during in vitro digestion, implying their vital role in the bioactivity of CS. In summary, the results obtained sustain the potential of CS to be valorized as a valuable antioxidant ingredient, able to regulate oxidative stress, and providing neuroprotection. Nonetheless, a higher fraction of phenolics from CS (≈80%) was not recovered upon digestion, which confirms the potential benefits of encapsulation techniques to enhance the bioaccessibility of these bioactive molecules. Therefore, future perspectives could lay emphasis on selecting the most suitable extraction technique to improve recovery of phenolics from CS and incorporate them into functional foods or dietary supplements using encapsulation techniques to enhance their bioaccessibility upon digestion. In addition, the composition of CS in condensed phenolic compounds, soluble and insoluble fiber, proanthocyanidins, and other insoluble polymeric phenolic compounds, should be explored post-in vitro digestion, as

there has been a lack until now of comprehensive understanding of their metabolization and biotransformation. To the best of our knowledge, this study establishes a fundamental framework for the exploitation of CS as an active ingredient for the development of food and nutraceutical products.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/separations10090471/s1, Table S1: Putative annotation of compounds in undigested CS and its digested fractions after oral, gastric, and intestinal digestion by LC-ESI-LTQ-Orbitrap-MS regarding untargeted metabolomic profiling. O, oral digest. G, gastric digest. I, intestinal digest.

Author Contributions: Conceptualization, F.R.; methodology, D.P., A.S.F., J.L.-C., E.P.L.-S., A.V.-Q., and F.R.; software, D.P., J.L.-C. and F.R.; validation, F.R.; formal analysis, D.P., A.S.F., E.P.L.-S., J.L.-C. and F.R.; investigation, D.P., A.S.F., E.P.L.-S. and J.L.-C.; resources, R.M.L.-R., C.D.-M. and F.R.; data curation, A.V.-Q. and F.R.; writing—original draft preparation, D.P.; writing—review and editing, J.L.-C., E.P.L.-S., A.V.-Q. and F.R.; visualization, R.M.L.-R. and C.D.-M.; supervision, F.R.; project administration, F.R.; funding acquisition, R.M.L.-R., C.D.-M. and F.R. All authors have read and agreed to the published version of the manuscript.

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