

1 **Antioxidant potential of chestnut, *Castanea sativa* L., and**
2 **almond, *Prunus dulcis* L., by-products**

3
4
5
6 **João C.M. Barreira ^{a,b}, Isabel C. F. R. Ferreira ^{a,*}, M. Beatriz P. P. Oliveira ^b and**
7 **José Alberto Pereira ^a**

8
9
10 ^a*CIMO/Escola Superior Agrária, Instituto Politécnico de Bragança, Campus de Santa*
11 *Apolónia, Apartado 1172, 5301-855 Bragança, Portugal.*

12 ^b*REQUIMTE/Serviço de Bromatologia, Faculdade de Farmácia da Universidade do Porto,*
13 *Rua Aníbal Cunha, 164, 4099-030 Porto, Portugal.*

14
15
16
17
18
19
20 *Author to whom correspondence should be addressed (e-mail: iferreira@ipb.pt, telephone
21 +351-273303219, fax +351-273-325405).

22 **ABSTRACT**

23 The antioxidant properties of almond green husks (Cvs. *Duro Italiano*, *Ferraduel*, *Ferranhês*,
24 *Ferrastar* and *Orelha de Mula*), chestnut skins and chestnut leaves (Cvs. *Aveleira*, *Boa*
25 *Ventura*, *Judia* and *Longal*) were evaluated through several chemical and biochemical assays
26 in order to provide a novel strategy to stimulate the application of waste products as new
27 suppliers of useful bioactive compounds, namely antioxidants. All the assayed by-products
28 revealed good antioxidant properties, with very low EC₅₀ values (lower than 380 µg/mL),
29 particularly for lipid peroxidation inhibition (lower than 140 µg/mL). The total phenols and
30 flavonoid contents were also obtained. The correlation between these bioactive compounds
31 and DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging activity, reducing power,
32 inhibition of β-carotene bleaching and inhibition of lipid peroxidation in pig brain tissue
33 through formation of thiobarbituric acid reactive substances (TBARS), was also obtained.
34 Although, all the assayed by-products proved to have a high potential of application in new
35 antioxidants formulations, chestnut skins and leaves demonstrated better results.

36

37

38 **KEYWORDS:** Chestnut, Almond, By-products, Antioxidant activity, total phenols.

39

40 INTRODUCTION

41 The interest in polyphenolic antioxidants has increased remarkably in the last decade because
42 of their elevated capacity in scavenging free radicals associated with various diseases (Silva et
43 al., 2007). Some studies indicate that dietary polyphenols have a protective effect against
44 coronary heart disease (Weisburger, 1999; Engler & Engler, 2006), cancer (Fang et al., 2002;
45 Nichenametla et al., 2006), neurodegenerative diseases (Lau et al., 2005) and osteoporosis
46 (Weaver & Cheong, 2005).

47 Chestnut and almond are important sources of phenolic compounds. Particularly chestnut
48 fruits (Ribeiro et al., 2007), chestnut leaves (Calliste et al., 2005), almond hulls (Sang et al.,
49 2002; Takeoka & Dao, 2003), almond skins (Sang et al., 2002), almond shells (Pinelo et al.,
50 2004), and almond fruits (Milbury et al., 2006) contain those compounds.

51 Portugal is one of the most important chestnut producers, with nearly 25% of European
52 production. Trás-os-Montes region represent 75.8% of Portuguese chestnut crops and 84.9%
53 of chestnut orchards area (23338 ha). The best development conditions are found at altitudes
54 higher than 500 m and winter low temperatures, as in the “Terra Fria Transmontana” region
55 (Northeast of Portugal) in which **12500 ha** are used for chestnut cultivation (Ribeiro et al.,
56 2007). Almond is also an important product, with **24522** crops spread trough **36530 ha**. This
57 culture is mainly located in Algarve and “Terra Quente Transmontana” ([http://portal.min-](http://portal.min-agricultura.pt/portal/page/portal/MADRP/PT)
58 [agricultura.pt/portal/page/portal/MADRP/PT](http://portal.min-agricultura.pt/portal/page/portal/MADRP/PT); Cordeiro & Monteiro, 2001; Martins et al.,
59 2003). Accordingly, it would be very important to perform a complete characterization of the
60 antioxidant potential of different by-products originated in these Portuguese crops or by their
61 industrial applications. Due to the multifunctional characteristics of phytochemicals, the
62 antioxidant efficacy of a plant extract is best evaluated based on results obtained by
63 commonly accepted assays, taking into account different oxidative conditions, system
64 compositions, and antioxidant mechanisms (Weisburger, 1999).

65 In the present work, the antioxidant properties of almond green husks (*Cvs. Duro Italiano,*
66 *Ferraduel, Ferranhês, Ferrastar and Orelha de Mula*), chestnut skins and chestnut leaves
67 (*Cvs. Avelreira, Boa Ventura, Judia and Longal*) were evaluated through several chemical and
68 biochemical assays: DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging activity,
69 reducing power, inhibition of β -carotene bleaching and thiobarbituric acid reactive substances
70 (TBARS) formation in brain cells. The whole extracts were used since they contain different
71 compounds that can act synergistically, constituting a benefit in comparison to individual
72 compounds (Pellegrini et al., 2006; Pereira et al., 2006).

73 The evaluation of the antioxidant properties stands as an interesting and valuable task,
74 particularly for finding new sources for natural antioxidants and nutraceuticals, providing a
75 novel strategy to stimulate the application of these by-products as new suppliers of useful
76 bioactive compounds.

77

78 **MATERIALS AND METHODS**

79

80 **Standards and Reagents**

81 Standards BHA (2-*tert*-butyl-4-methoxyphenol), TBHQ (tert-butylhydroquinone), L-ascorbic
82 acid, α -tocopherol, gallic acid and (+)-catechin were purchase from Sigma (St. Louis, MO,
83 USA). 2,2-diphenyl-1-picrylhydrazyl (DPPH) was obtained from Alfa Aesar (Ward Hill, MA,
84 USA). All other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO, USA).
85 Methanol was obtained from Pronalab (Lisbon, Portugal). Water was treated in a Mili-Q
86 water purification system (TGI Pure Water Systems, USA).

87

88 **Samples and sample preparation**

89 Chestnut tree leaves and chestnut skins were obtained from four different cultivars (Cvs.
90 *Aveleira*, *Boa Ventura*, *Judia* and *Longal*) and collected from orchards located in Vinhais
91 (*Trás-os-Montes*), in the Northeast side of Portugal. Leaves were collected monthly from June
92 to October and used miscellaneously (equal number of leaves for each month), and fruits were
93 collected in October and November. These samples were obtained during the crop year of
94 2006. Almond husks were obtained from five different cultivars (*Duro Italiano*, *Ferraduel*,
95 *Ferranhês*, *Ferrastar* and *Orelha de Mula*) and collected in August-September 2006 in
96 orchards located in Southwest Trás-os-Montes, Northeast Portugal. Selected plants are not
97 irrigated and no phytosanitary treatments were applied.

98 Chestnut leaves and almond husks were dried at 65 °C until constant weight was achieved and
99 kept at -20 °C until further use. Outer and inner skins were removed from chestnuts and
100 submitted to a roasting process conducted at 250 °C in a muffle furnace (ECF 12/22, Lenton
101 Thermal Designs Limited) for 15 minutes, to mimetize industrial practices. Inner and outer
102 skins were assayed together maintaining the individual proportion found for each variety
103 (outer skins represent a higher chestnut weight percentage, when compared with inner skins).

104 For antioxidant compounds extraction, a fine dried powder (20 mesh) of sample was extracted
105 using water, under magnetic stirring (150 rpm) at room temperature during 1h. The extracts
106 were filtered through Whatman nº 4 paper under reduced pressure, frozen at – 80 °C and then
107 lyophilized (Ly-8-FM-ULE, Snijders) at -80 to -90 °C under a reduced pressure of ~0.045
108 mbar. All the samples were redissolved in water at a concentration of 50 mg/mL, diluted to
109 final concentrations and analysed for their contents in polyphenols and flavonoids, DPPH
110 radical scavenging activity, reducing power, inhibition of β -carotene bleaching and inhibition
111 of lipid peroxidation.

112

113 **Determination of antioxidants content**

114 Content of total phenols in the extracts was estimated by a colorimetric assay based on
115 procedures described by Singleton and Rossi (Singleton & Rossi, 1965) with some
116 modifications. Basically, 1 mL of sample was mixed with 1 mL of Folin and Ciocalteu's
117 phenol reagent. After 3 min, 1 mL of saturated sodium carbonate solution was added to the
118 mixture and adjusted to 10 mL with distilled water. The reaction was kept in the dark for 90
119 min, after which the absorbance was read at 725 nm (Analytik Jena 200-2004
120 spectrophotometer). Gallic acid was used for constructing the standard curve (0.01-0.4 mM, y
121 $= 2.94848x - 0.09211$, $R^2 = 0.99914$) and the results were expressed as mg of gallic acid
122 equivalents/g of extract (GAEs).

123 Flavonoid contents in the extracts were determined by a colorimetric method described by Jia
124 *et al.* (1999) with some modifications. The extract (250 μ L) was mixed with 1.25 mL of
125 distilled water and 75 μ L of a 5% NaNO₂ solution. After 5 min, 150 μ L of a 10% AlCl₃.H₂O
126 solution was added. After 6 min, 500 μ L of 1M NaOH and 275 μ L of distilled water were
127 added to prepare the mixture. The solution was mixed well and the absorbance was read at
128 380 nm, 425 nm and 510 nm, in order to compare the results. (+)-Catechin (0.250-2.500 mM)
129 was used to calculate the standard curves, ($y=2.4553x - 0.1796$, $R^2=0.997$, at 340 nm,
130 $y=0.7376x - 0.0131$, $R^2=0.997$, at 425 nm, $y=0.5579x - 0.0494$, $R^2=0.992$, at 510 nm, and the
131 results were expressed as mg of (+)-catechin equivalents (CEs) per g of extract.

132

133 **DPPH radical-scavenging activity**

134 Various concentrations of extracts (0.3 mL) were mixed with 2.7 mL of methanolic solution
135 containing DPPH radicals (6×10^{-5} mol/L). The mixture was shaken vigorously and left to
136 stand for 60 min in the dark (until stable absorbance values were obtained). The reduction of
137 the DPPH radical was determined by reading the absorbance at 517 nm. The radical
138 scavenging activity (RSA) was calculated as a percentage of DPPH discolouration using the

139 equation: $\% \text{ RSA} = [(A_{\text{DPPH}} - A_{\text{S}}) / A_{\text{DPPH}}] \times 100$, where A_{S} is the absorbance of the solution
140 when the sample extract has been added at a particular level, and A_{DPPH} is the absorbance of
141 the DPPH solution (Barreira et al., 2008). The extract concentration providing 50% of radicals
142 scavenging activity (EC_{50}) was calculated from the graph of RSA percentage against extract
143 concentration. BHA and α -tocopherol were used as standards.

144

145 **Reducing power**

146 Several concentrations of extracts (2.5 mL) were mixed with 2.5 mL of 200 mmol/L sodium
147 phosphate buffer and 2.5 mL of potassium ferricyanide (1%). The mixture was incubated at
148 50 °C for 20 min. After 2.5 mL of trichloroacetic acid (10% w/v) were added, and the mixture
149 was centrifuged at 1000 rpm for 8 min (Centorion K24OR- 2003 refrigerated centrifuge). The
150 upper layer (5 mL) was mixed with 5 mL of deionised water and 1mL of ferric chloride
151 (0.1%), and the absorbance was measured spectrophotometrically at 700 nm (Barreira et al.,
152 2008). The extract concentration providing 0.5 of absorbance (EC_{50}) was calculated from the
153 graph of absorbance at 700 nm against extract concentration. BHA and α -tocopherol were
154 used as standards.

155

156 **Inhibition of β -carotene bleaching**

157 The antioxidant activity of aqueous extracts was evaluated by the β -carotene linoleate model
158 system. A solution of β -carotene was prepared by dissolving 2 mg of β -carotene in 10 mL of
159 chloroform. 2 mL of this solution were pipetted into a 100 mL round-bottom flask. After the
160 removal of the chloroform at 40°C under vacuum, 40 mg of linoleic acid, 400 mg of Tween
161 80 emulsifier, and 100 mL of distilled water were added to the flask with vigorous shaking.
162 Aliquots (4.8 mL) of this emulsion were transferred into different test tubes containing 0.2
163 mL of different concentrations of chestnut extracts. The tubes were shaken and incubated at

164 50°C in a water bath. As soon as the emulsion was added to each tube, the zero time
165 absorbance was measured at 470 nm. Absorbance readings were then recorded at 20-min
166 intervals until the control sample had changed colour. A blank, devoid of β -carotene, was
167 prepared for background subtraction. Lipid peroxidation (LPO) inhibition was calculated
168 using the following equation: LPO inhibition = (β -carotene content after 2h of assay/initial β -
169 carotene content) \times 100 (Barreira et al., 2008). The extract concentration providing 50%
170 antioxidant activity (EC₅₀) was calculated from the graph of antioxidant activity percentage
171 against extract concentration. TBHQ was used as standard.

172

173 **Inhibition of lipid peroxidation using thiobarbituric acid reactive substances (TBARS)**

174 Brains were obtained from pig (*Sus scrofa*) of body weight ~150 kg, dissected and
175 homogenized with a Polytron in ice-cold Tris-HCl buffer (20 mM, pH 7.4) to produce a 1:2
176 (w/v) brain tissue homogenate which was centrifuged at 3000g for 10 min. An aliquot (0.1
177 mL) of the supernatant was incubated with the extracts (0.2 mL) in the presence of FeSO₄ (10
178 μ M, 0.1 mL) and ascorbic acid (0.1 mM, 0.1 mL) at 37°C for 1 h. The reaction was stopped
179 by the addition of trichloroacetic acid (28% w/v, 0.5 mL), followed by thiobarbituric acid
180 (TBA, 2%, w/v, 0.38 mL), and the mixture was then heated at 80 °C for 20 min. After
181 centrifugation at 3000g for 10 min to remove the precipitated protein, the colour intensity of
182 the TBARS in the supernatant was measured by its absorbance at 532 nm. The inhibition ratio
183 (%) was calculated using the following formula: Inhibition ratio (%) = [(A - B)/A] \times 100%,
184 where A and B were the absorbance of the control and the compound solution, respectively
185 (Barreira et al., 2008). The extract concentration providing 50% lipid peroxidation inhibition
186 (EC₅₀) was calculated from the graph of antioxidant activity percentage against extract
187 concentration. BHA was used as standard.

188

189 **Statistical analysis**

190 For all the experiments three samples were analysed and all the assays were carried out in
191 triplicate. The results are expressed as mean values and standard error or standard deviation
192 (SD). The differences between the different extracts were analyzed using one-way analysis of
193 variance (ANOVA) followed by Tukey's honestly significant difference post hoc test with $\alpha =$
194 0.05, coupled with Welch's statistic. The regression analysis between total phenols or
195 flavonoid contents, and EC₅₀ values for antioxidant activity used the same statistical package.
196 These treatments were carried out using SPSS v. 16.0 program.

197

198 **RESULTS AND DISCUSSION**

199 **Table 1** presents extraction yields (expressed as w/w percentages), total phenols and
200 flavonoids content (mg/g of extract) obtained for chestnut and almond by-products. The
201 results are presented for each single variety in order to analyse possible differences. However,
202 and regarding the aim of this work, the results obtained for each by-product, as presented in
203 the bottom of the table, are the most significant, once it would be difficult to obtain supplies
204 of these by-products selected by variety. Among all of the extracts analyzed, an interesting
205 content of total phenols (from 228 to 859 mg/g) was detected with mean values of 592 mg/g
206 for almond husk, 413 mg/g for chestnut leaf and 710 mg/g for chestnut skins. The marked
207 differences of the results obtained for Longal leaf when compared with our previous study
208 ([Barreira et al., 2008](#)) can be explained on the basis of three different factors. First, the leaves
209 used in our previous work presented a higher ripeness state, second, they were utilized in
210 fresh (a drying step was not conducted), and finally the extraction procedure was conducted at
211 water boiling temperature. These results revealed the high potential of the assayed by-
212 products as new sources of antioxidant compounds. Extraction yields were generally low, but
213 their bioactivity indicates that the extraction procedure was effective, considering that the

214 objective was to achieve a clean extract. Despite this consideration, not all cases revealed a
215 relationship between extracted mass and total phenols content. Actually, extracts obtained
216 with chestnut skins proved to be the most uncontaminated, promoting it as the more adequate
217 by-products, considering the posterior purifying processes. Likewise, this observation could
218 probably be explained by a higher amount of other polar compounds in chestnut leaves and
219 almond husks.

220

221 **Figures 1 to 4** show the antioxidant activity of the extracts examined as a function of their
222 concentration. Several biochemical assays were used to screen the antioxidant properties:
223 inhibition of β -carotene bleaching (by neutralizing the linoleate-free radical and other free
224 radicals formed in the system which attack the highly unsaturated β -carotene models),
225 inhibition of lipid peroxidation in brain tissue (measured by the colour intensity of MDA-
226 TBA complex), scavenging activity on DPPH radicals (measuring the decrease in DPPH
227 radical absorption after exposure to radical scavengers) and reducing power (measuring the
228 conversion of a Fe^{3+} /ferricyanide complex to the ferrous form). The assays were carried out
229 using whole extracts instead of individual compounds, once additive and synergistic effects of
230 phytochemicals in fruits and vegetables are responsible for their potent bioactive properties
231 and the benefit of a diet rich in fruits and vegetables is attributed to the complex mixture of
232 phytochemicals present in whole foods (Liu, 2003). This enhances the advantages of natural
233 phytochemicals over single antioxidants when they are used to achieve health benefits.

234 Analysis of **figures 1 to 4** revealed that antioxidant activity increased with the concentration,
235 being obtained very good results even at low extract concentrations, especially for TBARS
236 assay.

237 The bleaching inhibition, measured by the peroxidation of β -carotene, is presented in **figure**
238 **1**. The linoleic acid free radical attacks the highly unsaturated β -carotene model. The presence

239 of different antioxidants can hinder the extent of β -carotene-bleaching by neutralizing the
240 linoleate-free radical and other free radicals formed in the system (Jayaprakasha et al., 2001).
241 Hence, the absorbance diminishes fast in samples without antioxidant, whereas in the
242 presence of an antioxidant, they maintain their colour, and thus absorbance, for a longer time.
243 Bleaching inhibition in the presence of different extracts increased with concentration and
244 proved to be very good. At 500 $\mu\text{g/mL}$, all the extracts presented inhibition percentages
245 superior to 65%, except in the cases of Orelha de Mula husk, a very good result once that the
246 antioxidant activity of TBHQ standard reached 82.2% only at 2 mg/ml. It is expectable that
247 the antioxidative components in the chestnut extracts reduce the extent of β -carotene
248 destruction by neutralizing the linoleate free radical and other free radicals formed in the
249 system. It became clear that chestnut derived by-products revealed higher efficiency in this
250 antioxidant activity biochemical assay when compared with almond by-products.

251 Inhibition of lipid peroxidation was evaluated using thiobarbituric acid reactive substances
252 (TBARS). When oxidation processes occur, a pinkish solution is formed. If antioxidant
253 compounds are present in the system, the formation of the substances responsible for the
254 coloration is prevented. As it can be easily understood after **figure 2** observation, the capacity
255 of inhibition of lipid peroxidation is proportional to the extract concentration. This method
256 revealed very high inhibition percentages at extremely low concentrations. All extracts
257 showed inhibition percentages superior to 60% at concentrations of 100 $\mu\text{g/mL}$, except for
258 Ferraduel husk and Judia leaf. Generally, chestnut skins and almond husks extracts proved to
259 be better inhibitors in this model.

260 The radical scavenging activity (RSA) values were expressed as the ratio percentage of
261 sample absorbance decrease and the absorbance of DPPH solution in the absence of extract at
262 517 nm. From the analysis of **figure 3**, we can conclude that the scavenging effects of all
263 extracts on DPPH radicals increased with the concentration increase and were remarkably

264 good, with RSA percentages superior to 90% at 500 µg/mL for almost all the extracts, except
265 for Aveleira and Judia leaves and Ferraduel and Ferranhês husks, again better than the
266 scavenging effects of some usual standards like BHA (96% at 3.6 mg/ml) and α -tocopherol
267 (95% at 8.6 mg/ml).

268 Like in the other assays previously referred, the reducing power increased with concentration,
269 and the values obtained for all the extracts were very good (**figure 4**). At 250 µg/mL, the
270 absorbance values were higher than 0.5 for all extracts, with the exception of Judia leaf and
271 Ferraduel and Orelha de Mula husks, proving once more to have much more high antioxidant
272 activity than some common standards (reducing powers of BHA at 3.6 mg/ml and a-
273 tocopherol at 8.6 mg/ml were only 0.12 and 0.13, respectively). The extracts obtained with
274 chestnut skins revealed better reducing properties. This difference could be explained by the
275 presence of high amounts of reductones, which have been associated with antioxidant action
276 due to breaking the free radical chain by donating a hydrogen atom ([Shimada et al., 1992](#)).

277 **Table 2** shows antioxidant activity EC₅₀ values of chestnut and almond by-products extracts
278 measured by different biochemical assays. In the lower part of the table these results are
279 represented for each one of the by-products. Overall, chestnut skins revealed better
280 antioxidant properties (significantly lower EC₅₀ values, $p < 0.05$). The EC₅₀ values obtained
281 for these extracts were excellent (less than 110 µg/mL, average value), particularly for LPO
282 inhibition (less than 40 µg/mL, average value). However, chestnut leaves (less than 220
283 µg/mL in average, for all assays) and almond husks (less than 260 µg/mL in average, for all
284 assays) also revealed very good antioxidant activity.

285 The obtained results are generally in agreement with the total phenol and flavonoid contents
286 determined for each sample and showed in **table 1**. The EC₅₀ values obtained for lipid
287 peroxidation inhibition were better than for reducing power, scavenging effects on DPPH

288 radicals and β -carotene bleaching inhibition caused by linoleate free radical, which were
289 similar.

290 Other tree nuts had demonstrate their potential antioxidant activity namely walnuts ([Anderson
291 et al., 2001](#); [Fukuda et al., 2004](#)) and hazelnuts ([Alasalvar et al., 2006](#); [Sivakumar &
292 Bacchetta, 2005](#)). Nevertheless, those studies were carried out with extracts from the fruits.

293 In previous works ([Barreira et al., 2008](#); [Barros et al., 2007](#); [Sousa et al., 2008](#)) we observed a
294 significantly negative linear correlation between the total phenols content and EC_{50}
295 antioxidant activity values. This negative linear correlation proves that the samples with
296 highest total phenols content show lower EC_{50} values, confirming that phenols are likely to
297 contribute to the antioxidant activity of the extracts, as it has been reported in other species
298 ([Velioglu et al., 1998](#)). The flavonoids contents were also correlated with EC_{50} scavenging
299 capacity values with similar correlation coefficients values. Furthermore, approximately half
300 of the results showed statistical significance, as it can be seen in **table 3**. This may represent
301 an important tool to predict this kind of bioactivity just by quantifying phenols.

302 In conclusion, all the assayed by-products revealed good antioxidant properties, with very low
303 EC_{50} values, particularly for lipid peroxidation inhibition, and might provide a novel strategy
304 to stimulate the application of waste products as new suppliers of useful bioactive
305 compounds, particularly antioxidants. This represents an additional advantage since almond
306 and chestnut are important products, with high economic value, which originate high amounts
307 of the studied by-products.

308

309 **Acknowledgements**

310 The authors are grateful to Foundation for Science and Technology (Portugal) for financial
311 support to J.C.M. Barreira (SFRH/BD/29060/2006) and INTERREG IIIA project PIREFI.

312

313 **References**

- 314 Alasalvar C., Karamaca M., Amarowicz R. and Shahidi F. (2006). Antioxidant and antiradical
315 activities in extracts of hazelnut kernel (*Corylus avellana* L.) and hazelnut green leafy cover.
316 Journal of Agriculture and Food Chemistry 54: 4826-4832.
- 317 Anderson K.J., Teuber S.S., Gobeille A., Cremin P., Waterhouse A.L. and Steinberg F.M.
318 (2001). Walnut polyphenolics inhibit in vitro human plasma and LDL oxidation. The Journal
319 of Nutrition 131: 2837-2842.
- 320 Barreira J.C.M., Ferreira I.C.F.R., Oliveira M.B.P.P. and Pereira J.A. (2008). Antioxidant
321 activity of the extracts from chestnut flower, leaf, skins and fruit. Food Chemistry 107: 1106-
322 1113.
- 323 Barros L., Baptista P. and Ferreira I.C.F.R. (2007). Effect of *Lactarius piperatus* fruiting body
324 maturity stage on antioxidant activity measured by several biochemical assays. Food and
325 Chemical Toxicology 45: 1731-1737.
- 326 Calliste C.-A., Trouillas P., Allais D.-P. and Duroux J.-L. (2005). *Castanea sativa* Mill.
327 leaves as new sources of natural antioxidant: an electronic spin resonance study. Journal of
328 Agriculture and Food Chemistry 53: 282-288.
- 329 Cordeiro V. and Monteiro A. (2001). Almond growing in Trás-os-Montes region (Portugal).
330 Acta Horticulturae 591: 161-165.
- 331 Engler M.B. and Engler M.M. (2006). The emerging role of flavonoid-rich cocoa and
332 chocolate in cardiovascular health and disease. Nutrition Reviews 64: 109-118.
- 333 Fang Y.-Z., Yang S. and Wu G. (2002). Free radicals, antioxidants, and nutrition. Nutrition
334 18: 872-879.
- 335 Fukuda T., Ito H. and Yoshida T. (2004). Effect of the walnut polyphenol fraction on
336 oxidative stress in type 2 diabetes mice. BioFactors 21: 251-253.

337 Jayaprakasha G.K., Singh R.P. and Sakariah K.K. (2001). Antioxidant activity of grape seed
338 (*Vitis vinifera*) extracts on peroxidation models in vitro. Food Chemistry 73: 285-290.

339 Jia Z., Tang M. and Wu J. (1999). The determination of flavonoid contents in mulberry and
340 their scavenging effects on superoxide radicals. Food Chemistry 64: 555-559.

341 Lau F.C., Shukitt-Hale B.J. and Joseph A. (2005). The beneficial effects of fruit polyphenols
342 on brain aging. Neurobiology of Aging 26: 128-132.

343 Liu R.H. (2003). Health benefits of fruits and vegetables are from additive and synergistic
344 combination of phytochemicals. American Journal of Clinical Nutrition 78: 517S-520S-9.

345 Martins M., Tenreiro R. and Oliveira M.M. (2003). Genetic relatedness of Portuguese almond
346 cultivars assessed by RAPD and ISSR markers. Plant Cell Reports 22: 71-78.

347 Milbury P.E., Chen C.-Y., Dolnikowski G.G. and Blumberg J.B. (2006). Determination of
348 flavonoids and phenolics and their distribution in almonds. Journal of Agriculture and Food
349 Chemistry 54: 5027-5033.

350 Nichenametla S.N., Taruscio T.G., Barney D.L. and Exon J.H. (2006). A review of the effects
351 and mechanisms of polyphenolics in cancer. Critical Review in Food Science and Nutrition
352 46: 161-183.

353 Pellegrini N., Serafini M., Salvatore S., Del Rio D., Bianchi M. and Brighenti F. (2006). Total
354 antioxidant capacity of spices, dried fruits, nuts, pulses, cereals and sweets consumed in Italy
355 assessed by three different in vitro assays. Molecular Nutrition & Food Research 50: 1030-
356 1038.

357 Pereira J.A., Pereira A.P.G., Ferreira I.C.F.R., Valentão P., Andrade P.B., Seabra R.,
358 Estevinho L. and Bento A. (2006). Table olives from Portugal: phenolic compounds,
359 antioxidant potential and antimicrobial activity. Journal of Agriculture and Food Chemistry
360 54: 8425-8431.

361 Pinelo M., Rubilar M., Jerez M., Sineiro J. and Núñez M.J. (2004). Extraction of antioxidant
362 phenolics from almond hulls (*Prunus amygdalus*) and pine sawdust (*Pinus pinaster*). Food
363 Chemistry 85: 267-273.

364 Ribeiro B., Rangel J., Valentão P., Andrade P.B., Pereira J.A., Bolke H. and Seabra R.M.
365 (2007). Organic acids in two Portuguese chestnut (*Castanea sativa* Miller) varieties. Food
366 Chemistry 100: 504-508.

367 Sang S., Lapsley K., Jeong W.-S., Lachance P.A., Ho C.H. and Rosen R.T. (2002).
368 Antioxidative phenolic compounds isolated from almond skins (*Prunus amygdalus* Batsch).
369 Journal of Agriculture and Food Chemistry 50: 2459-2463.

370 Shimada K., Fujikawa K., Yahara K. and Nakamura T. (1992). Antioxidative properties of
371 xanthan on the autoxidation of soybean oil in cyclodextrin emulsion. Journal of Agriculture
372 and Food Chemistry 40: 945-948.

373 Silva E.M., Souza J.N.S., Rogez H., Rees J.F. and Larondelle Y. (2007). Antioxidant
374 activities and polyphenolic contents of fifteen selected plant species from the Amazonian
375 region. Food Chemistry 101: 1012-1018.

376 Singleton V.L. and Rossi J.A.Jr. (1965). Colorimetric of total phenolics with
377 phosphomolybdic-phosphotungstic acid reagents. American Journal of Enology and
378 Viticulture 16: 144-158.

379 Sivakumar G. and Bacchetta L. (2005). Determination of natural vitamin E from Italian
380 hazelnut leaves. Chemistry of Natural Compounds 41: 654-656.

381 Sousa A., Ferreira I.C.F.R., Barros L., Bento A. and Pereira J.A. (2008). Effect of solvent and
382 extraction temperatures on the antioxidant potential of traditional stoned table olives
383 “*alcaparras*”. LWT- Food Science and Technology 41: 739-745.

384 Takeoka G.R. and Dao L.T. (2003). Antioxidant constituents of almond (*Prunus dulcis* (Mill.)
385 D.A. Webb) hulls. Journal of Agriculture and Food Chemistry 51: 496-501.

386 Velioglu Y.S., Mazza G., Gao L. and Oomah B.D. (1998). Antioxidant activity and total
387 phenolics in selected fruits, vegetables, and grain products. *Journal of Agriculture and Food*
388 *Chemistry* 46: 4113-4117.

389 Weaver C.M. and Cheong J.M. (2005). Soy isoflavones and bone health: the relationship is
390 still unclear. *Journal of Nutrition* 135: 1243-1247.

391 Weisburger J. (1999). Mechanisms of action of antioxidants as exemplified in vegetables,
392 tomatoes and tea. *Food and Chemical Toxicology* 37: 943-948.

393 **Fig. 1.** Inhibition of β -carotene bleaching as a function of extracts concentration.

394

395 **Fig. 2.** Lipid peroxidation (LPO) inhibition as a function of extracts concentration.

396

397 **Fig. 3.** Radical Scavenging Activity (RSA) as a function of extracts concentration.

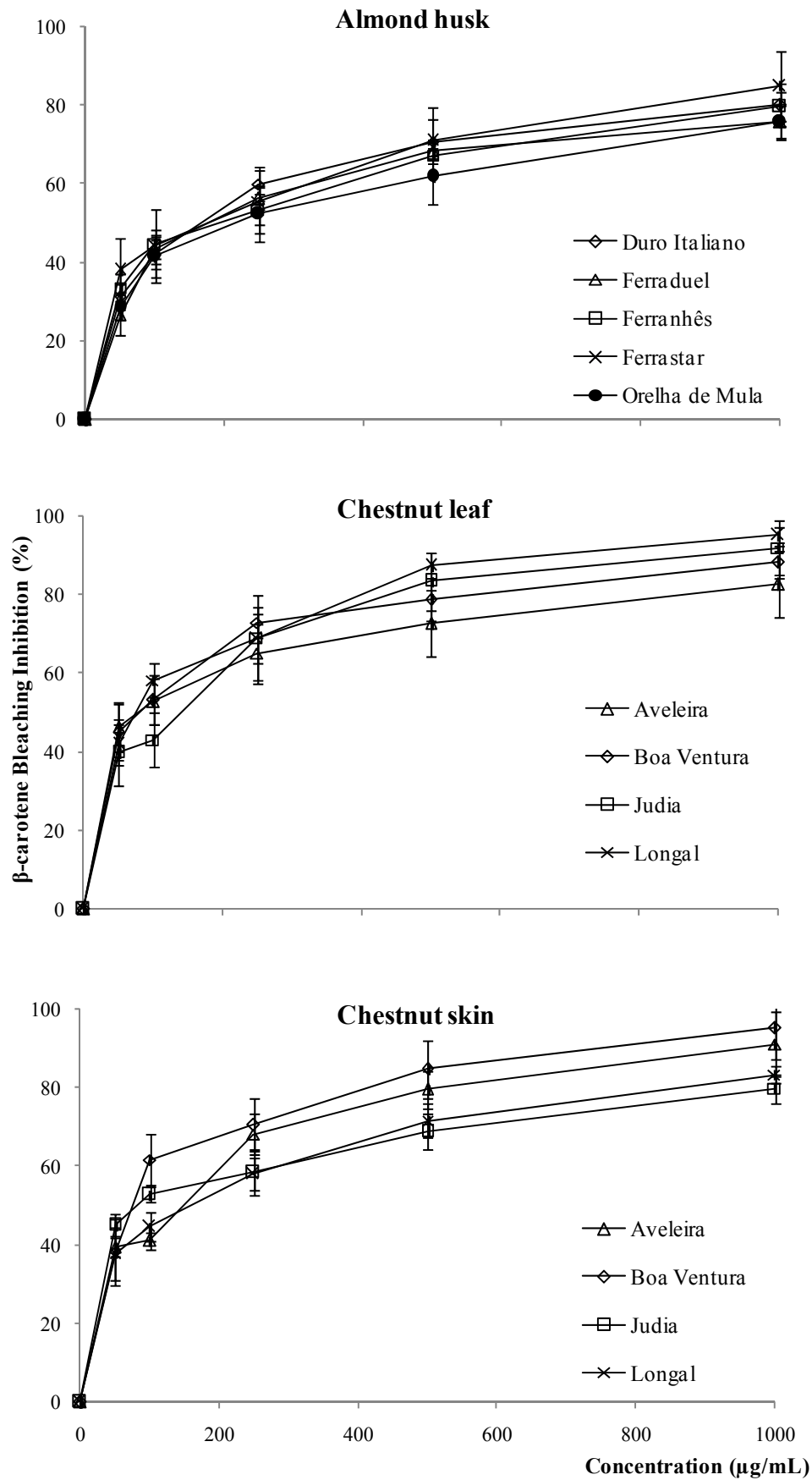
398

399 **Fig. 4.** Reducing power as a function of extracts concentration.

400

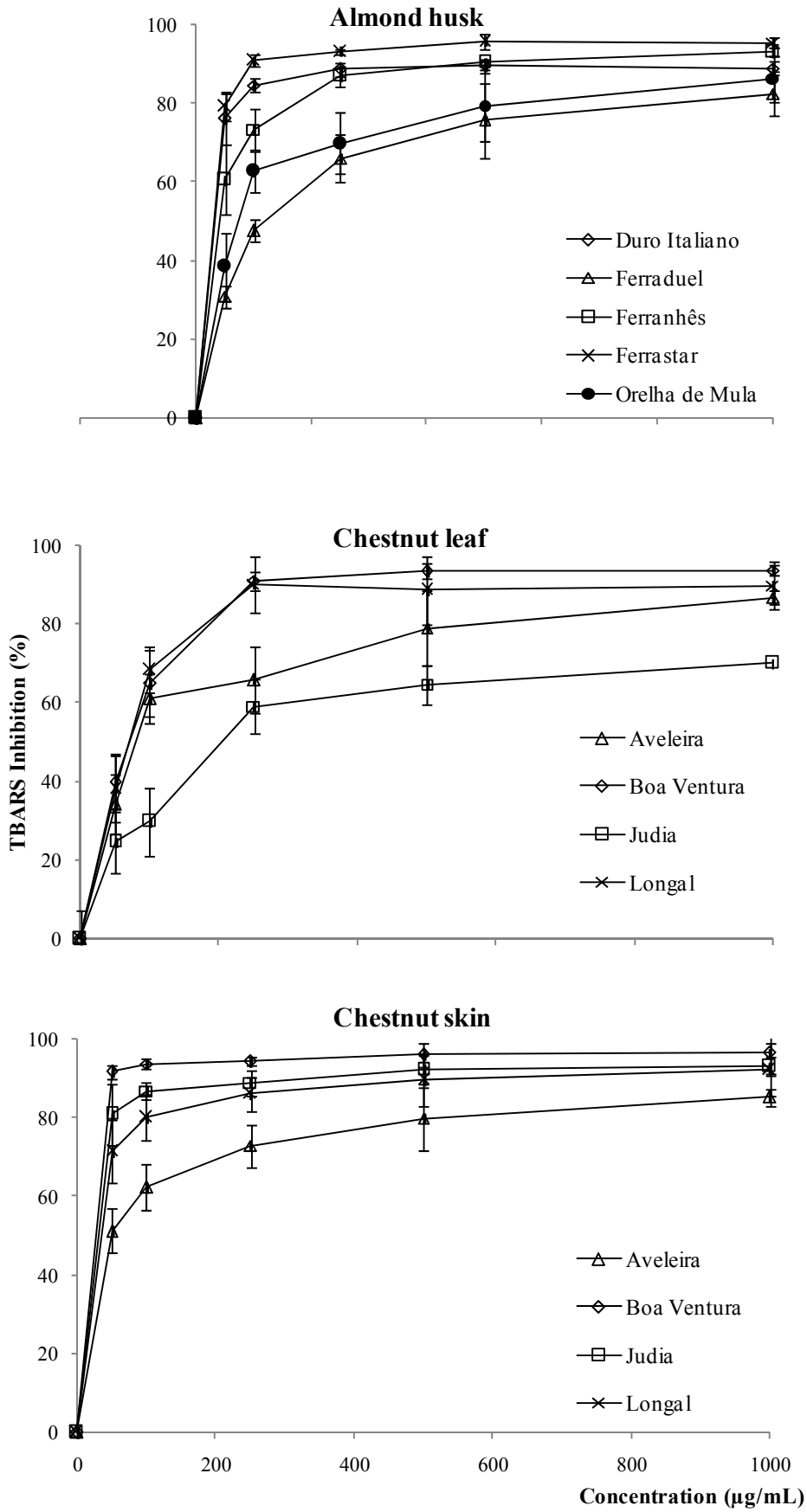
401

402



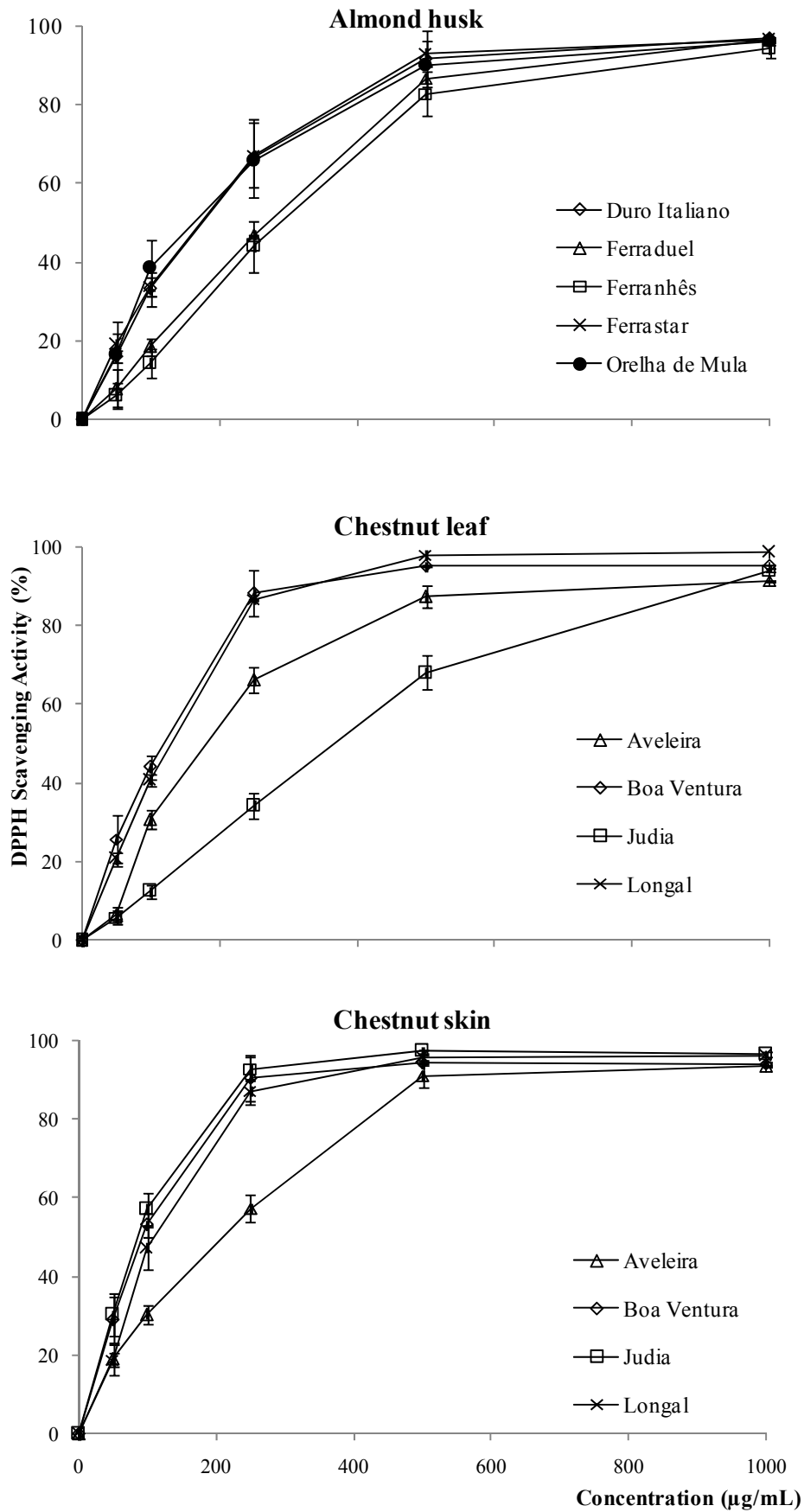
403

404 **Figure 1.**



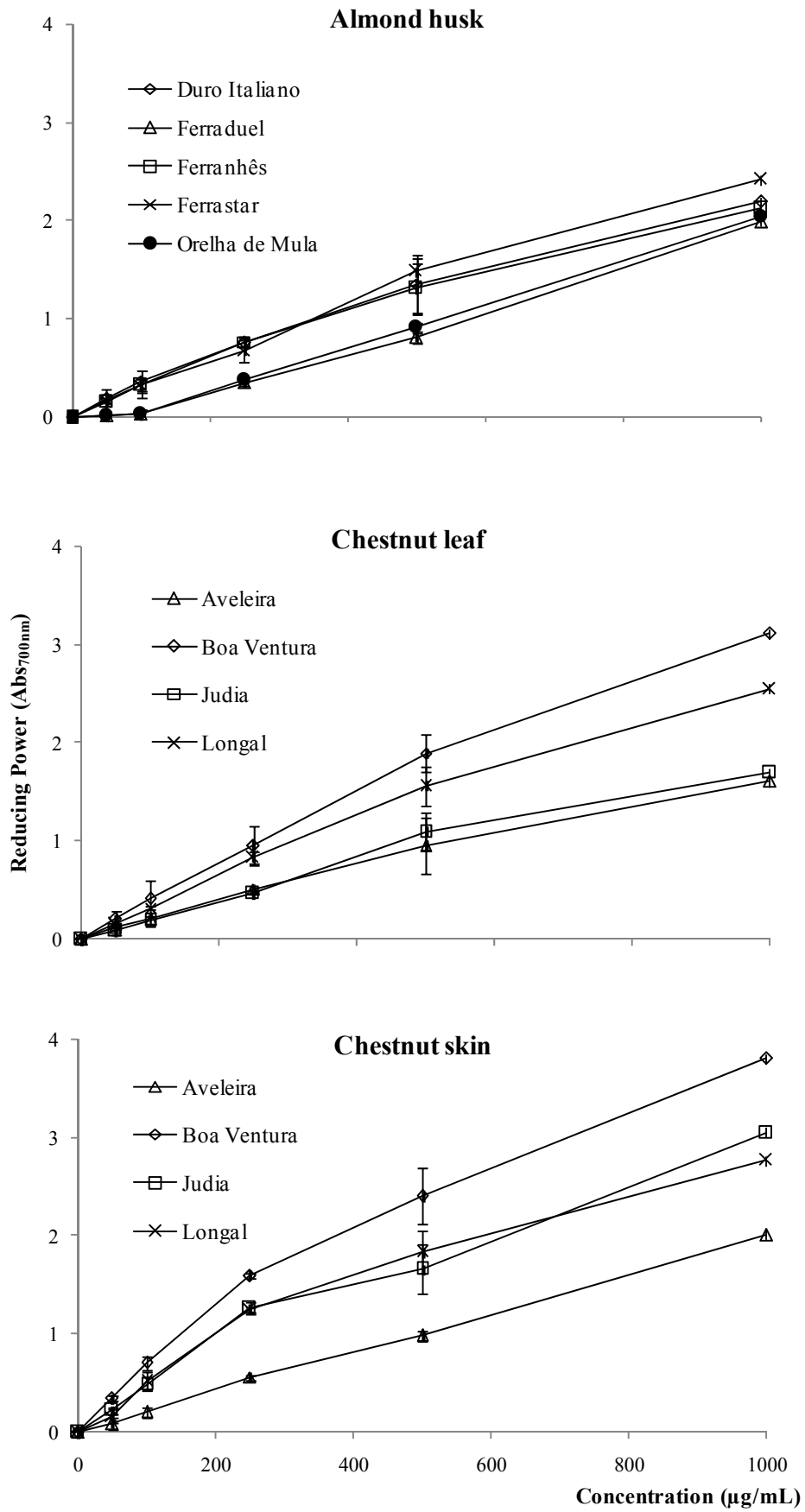
405

406 **Figure 2.**



407

408 **Figure 3.**



409

410 **Figure 4.**

411 **Table 1.** Extraction yields, content of total phenols and flavonoids in the extracts of chestnut
 412 and almond by-products. In each column and for each by product, different letters mean
 413 significant differences ($p < 0.05$).

		Extraction yield (%)	Total phenols (mg/g)	Flavonoids (mg/g)
Almond husk (AH)	Duro Italiano	17.65±1.02 c	777.21±18.78 b	237.20±2.52 b
	Ferraduel	14.14±0.60 c	304.79±22.06 e	70.48±3.61 e
	Ferranhês	27.49±2.11 a	378.70±9.42 d	130.68±5.91 c
	Ferrastar	22.58±1.18 b	859.07±74.50 a	284.61±12.06 a
	Orelha de Mula	22.81±1.55 b	639.75±33.91 c	116.88±19.49 d
Chestnut leaf (CL)	Aveleira	17.67±0.94 a	468.34±25.47 b	84.68±3.72 b
	Boa Ventura	15.62±0.93 bc	432.16±37.59 c	83.09±6.82 b
	Judia	17.08±0.62 ab	228.37±13.99 d	73.31±4.89 c
	Longal	13.73±0.49 c	522.98±23.82 a	90.39±5.57 a
Chestnut skin (CS)	Aveleira	7.17±0.29 b	533.81±30.90 c	49.92±1.93 d
	Boa Ventura	6.43±0.32 b	805.74±74.31 a	146.08±4.19 a
	Judia	12.59±0.84 a	757.95±67.51 b	98.10±6.62 b
	Longal	6.47±0.43 b	742.33±37.46 b	72.27±3.78 c
AH	\bar{x}	20.93±4.91 a	591.90±221.39 b	167.97±80.88 a
CL	\bar{x}	16.02±1.72 b	412.96±114.91 c	82.87±8.13 b
CS	\bar{x}	8.16±2.72 c	709.96±118.38 a	91.59±36.21 b

414

415

416

417

418

419 **Table 2.** EC₅₀ values (µg/mL) obtained in the antioxidant assays for chestnut and almond by-
 420 products and corresponding coefficients of variation (%). In each column and for each by
 421 product, different letters mean significant differences ($p < 0.05$).

422

		Bleaching inhibition	LPO inhibition	RSA	Reducing Power
Almond husk (AH)	Duro Italiano	227.37±18.44 c	29.20±2.65 d	175.03±11.42 c	206.96±20.63 c
	Ferraduel	284.91±17.52 a	103.52±6.78 a	216.37±14.15 a	376.30±27.67 a
	Ferranhês	250.23±18.83 b	39.95±3.63 c	209.22±14.61 a	218.11±21.06 c
	Ferrastar	211.37±9.25 d	28.11±1.15 d	176.82±12.34 c	169.85±4.53 d
	Orelha de Mula	276.77±10.53 a	74.15±3.61 b	190.33±4.53 b	306.46±22.13 b
Chestnut leaf (CL)	Aveleira	99.47±5.33 b	78.32±6.01 b	182.97±8.23 b	210.09±18.92 b
	Boa Ventura	99.09±5.37 b	71.54±5.86 c	161.34±9.08 c	215.62±8.87 b
	Judia	160.04±15.17 a	133.52±5.60 a	367.06±27.89 a	267.00±26.54 a
	Longal	64.14±3.76 c	69.04±3.53 c	129.91±5.02 d	152.38±2.39 c
Chestnut skin (CS)	Aveleira	151.27±15.55 a	49.07±4.83 a	159.99±15.37 a	117.58±12.71 a
	Boa Ventura	74.62±8.92 d	27.29±0.48 d	82.41±5.52 c	79.25±6.39 d
	Judia	86.07±7.16 c	30.47±2.05 c	86.52±7.77 c	104.61±8.22 b
	Longal	120.84±7.84 b	34.53±3.21 b	108.87±6.73 b	94.55±6.31 c
AH	\bar{x}	250.13±32.03 a	54.98±29.82 b	193.56±20.52 a	255.53±78.19 a
CL	\bar{x}	105.68±35.71 b	88.10±27.08 a	210.32±94.11 a	211.27±44.08 b
CS	\bar{x}	108.20±31.97 b	35.34±8.90 c	109.45±32.44 b	99.00±16.54 c

423

424 **Table 3.** Correlations established between total phenols and flavonoids with antioxidant
 425 activity EC₅₀ values.
 426

		Total phenols			Flavonoids		
		Equation <i>R</i> ²	<i>F</i>	<i>Sign.</i>	Equation <i>R</i> ²	<i>F</i>	<i>Sign.</i>
Almond husk	Bleaching inhibition	y = -0.0001x + 0.3086 0.584	4.218	n.s.	y = -0.0003x + 0.3073 0.937	44.610	**
	LPO inhibition	y = -0.0001x + 0.1096 0.463	2.590	n.s.	y = -0.0003x + 0.1080 0.733	18.238	n.s.
	RSA	y = -0.0001x + 0.2386 0.976	120.893	**	y = -0.0001x + 0.2245 0.774	10.269	*
	Reducing Power	y = -0.0002x + 0.3964 0.473	2.6886	n.s.	y = -0.0008x + 0.3942 0.769	9.979	n.s.
Chestnut leaf	Bleaching inhibition	y = -0.0003x + 0.2312 0.962	50.278	*	y = -0.0056x + 0.5686 0.990	208.436	*
	LPO inhibition	y = -0.0002x + 0.1825 0.927	25.419	*	y = -0.0040x + 0.4162 0.848	11.133	n.s.
	RSA	y = -0.0008x + 0.5452 0.955	42.044	*	y = -0.0143x + 1.3957 0.905	19.055	*
	Reducing Power	y = -0.0003x + 0.3507 0.857	12.020	n.s.	y = -0.065x + 0.7466 0.957	44.141	*
Chestnut skin	Bleaching inhibition	y = -0.0003x + 0.2949 0.830	9.738	n.s.	y = -0.0008x + 0.1800 0.866	12.890	n.s.
	LPO inhibition	y = -0.0001x + 0.0916 0.984	121.371	**	y = -0.0002x + 0.0537 0.742	5.736	n.s.
	RSA	y = -0.0003x + 0.3150 0.958	45.713	*	y = -0.0007x + 0.1759 0.708	4.851	n.s.
	Reducing Power	y = -0.0001x + 0.1811 0.741	5.731	n.s.	y = -0.0003x + 0.1299 0.741	5.727	n.s.

427 *, *p* ≤ 0.05 (significant correlation), **, *p* ≤ 0.01 (very significant correlation), ***, *p* ≤ 0.001 (extremely
 428 significant correlation), n.s., not significant correlation.
 429