1 *Article*

2 A bio-guided approach for the development of a chestnut-based 3 proanthocyanidin-enriched nutraceutical with potential anti-gastritis 4 properties

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14

15 Abstract

16 Gastritis is a widely spread inflammatory disease, mostly caused by *Helicobacter pylori* infection. 17 Release of IL-8 by the stomach epithelium is a hallmark of gastritis and contributes to the 18 amplification of the inflammatory state. Pharmacological modulation of IL-8 release is a strategy to 19 relieve gastric inflammation and prevent more severe clinical outcomes. In search of nutraceuticals 20 with potential anti-gastritis properties we used a bio-guided approach based on IL-8 secretion by 21 gastric cells to characterize extracts from the fruits of different chestnut varieties.

We found that the ability to inhibit IL-8 secretion correlated with the amount of proanthocyanidins and was associated to the not edible parts of chestnut in all the tested varieties. We also found that the anti-inflammatory activity is preserved upon mild thermal treatment and after *in vitro* simulated gastric digestion.

By combining a robust bio-guided approach with a comprehensive analysis of the tannin fraction of chestnut extracts, we provide evidence for the potential use of chestnut-based nutraceuticals in human gastritis. The bioactive components of chestnut fruits inhibit IL-8 secretion by impairing NF-κB signaling and by other mechanisms, thus opening new applications of proanthocyanidins for inflammation-based diseases.

Keywords: Chestnut; gastric inflammation; proanthocyanidins; interleukin-8

Abbreviations

Interleukin-8 (IL-8), Tumor necrosis factor alpha (TNFα), Protected Geographic Indication (PGI), Dulbecco's Modified Eagle's Medium/F12 (DMEM)/F12, Foetal bovine serum (FBS), 3,4,5-dimethylthiazol- 2-yl-2-5-diphenyltetrazolium bromide (MTT).

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1. Introduction

Gastritis is a very common inflammatory disease, mostly caused by *Helicobacter pylori* (*H. pylori*) infection [1]. This gram negative bacterium colonizes the gastric mucosa of over 80% of people in developing countries [2] and at least 50% of the world human population [3]. *H. pylori* infection induces a strong immune response in the host [4], which is characterized by the release of several

43 inflammatory cytokines in the gastric mucosa, including IL-8 and TNFα [5-8]. Gastric epithelial cells
44 exposed to cytokines, mostly TNFα and IL-1β, release IL-8, a potent chemokine, which promotes
45 neutrophil infiltration [9-11]. IL-8 secretion is a typical hallmark in *H. pylori*-induced gastritis [12],
46 and its release by gastric epithelial cells is strictly associated with the activation of NF-κB [9, 13], a
47 transcription factor involved in a multitude of patho-physiological processes, including
48 inflammation, cell growth, and proliferation [14-16].

49 Plants rich in tannins have a traditional use for treating gastric ulcer; moreover, tannins showed anti-
50 bacterial activity against *H. pylori* [17, 18], and inhibition of gastric IL-8 release, both *in vitro* and *in*
51 *vivo* [19, 20]. Epidemiological studies indicate that dietary consumption of proanthocyanidins
52 (condensed tannins) has beneficial effects on a variety of chronic diseases, including metabolic
53 syndrome, atherosclerosis, and cancer [21, 22]. Moreover, proanthocyanidins have been found
54 unchanged after in vitro simulated gastric digestion [19], and in vivo at gastric level [23, 24], thus
55 suggesting that the biological activity may occur *in situ*.

56 Chestnut tree (*Castanea sativa* Mill., sin. *Castanea vesca* Gaertn.) is a rich source of tannins, mostly in
57 leaves, wood and bark, whereas fruits, which are a good source of essential dietary nutrients, showed
58 lower levels of polyphenols [25]. In Italy, chestnuts from six geographical areas are regulated by
59 Protected Geographic Indication (PGI) under European Union law and the average production of
60 this fruit in the period 2003-2013 was close to 49,000 tons/year [26]. Despite high production of
61 chestnut and the traditional dietary consumption in several European countries, only limited data on
62 the tannin composition of fruits and their beneficial properties occur in the literature. Few studies
63 performed on chestnut industrial by-products reported high content of phenols and marked

64 antioxidant properties [27, 28]. Tannins were identified in fruits, although more precise details on

65 their chemical features were not reported [29-31].

66 The aim of this study was to characterize the anti-inflammatory properties of chestnut bioactive

67 compounds, taking into consideration several variables (chestnut fruit parts, chestnut varieties,

68 harvest year, chemical and thermal stability, etc). To this end we used a bio-guided approach based

69 on IL-8 secretion by AGS cells stimulated with TNFα, a simple but validated model of gastritis

70 induced by *H. pylori*.

71 **2. Materials and Methods**

72 *2.1. Materials*

73 Dulbecco's Modified Eagle's Medium/F12 (DMEM)/F12 (1:1), penicillin, streptomycin, L-

74 glutamine and trypsin-EDTA were from Gibco (Life Technologies Italia, Monza, Italy). Foetal bovine

75 serum (FBS), and disposable materials for cell culture were purchased by Euroclone (Euro- clone

76 S.p.A., Pero-Milan, Italy). Human adenocarcinoma cells (AGS, CRL-1739) were purchased from LGC

77 Standard S.r.l., Milano, Italy.

78 The reagent 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and curcumin

79 were from Sigma Aldrich (Milan, Italy). All reagents used for the biological assays were HPLC grade.

80 Human TNF*α* and Human IL-8 Elisa Development Kit were from Peprotech Inc. (London, UK). All

81 chromatographic solvents were HPLC grade or LC-MS grade for MS experiments. Acetonitrile,

82 methanol, ethanol, formic acid, hydrochloric acid, vanillin, and iron sulfate were from Sigma Aldrich

83 (Milan, Italy).

84 *2.2. Plant material and preparation of the extracts*

85 Chestnuts from five varieties (Venégon, Paié, Russirö, Verdésa and Piliscé) of *Castanea sativa*

86 Mill. were collected by the farmer consortium in the regional area of Campo dei Fiori (Varese, Italy).

Fresh fruits were maintained under vacuum at 4 °C until extraction. To obtain the extracts 2,5 grams of milled whole fruit were extracted twice with 50 mL of water (aqueous extract) or ethanol/water 50:50 (hydroalcoholic extract) for 4 and 16 hours, respectively, at room temperature under dark conditions. The mixture was filtered through Supervelox filter paper in order to remove plant debris; the extracts obtained were frozen with dry ice and alcohol and placed at −80 °C overnight, then lyophilized and maintained at −20 °C. In general, the yields of the aqueous and hydroalcoholic extracts of the same variety were comparable with the exception of Verdésa, whose hydroalcoholic yield was the half of the aqueous one.

Three parts of the fruits, endosperm (kernel) and the outer parts episperm (which directly covers the kernel) and pericarp (the woody part), were separated and extracted with hydroalcoholic solvent following the procedure previously described. The yield of the different parts varied according to the variety.

The yields (w/w) of each extraction were calculated as percentage of the dried extract weight in respect to the weight of the fresh starting material. ~~The yields of the extracts are reported in the supplementary materials (Table S1).~~

Commercially available flour, produced from the endosperm of dried chestnut (variety Venégon), and relative industrial by-product, constituted by episperm and pericarp, were extracted by hydroalcoholic solvent. Before proceeding with the biological evaluation, the extracts were dissolved in sterilized distilled water and DMSO (25%) at a concentration of 30 mg/mL, then stored in aliquots at −20 °C. For the biochemical analysis, the lyophilized samples (10 mg) were dissolved in 5 mL of a mixture of methanol/water (2/1).

2.3. Cytotoxicity

The integrity of the cell morphology before and after treatment was assessed by light microscope inspection. Cell viability was measured, after 6 h treatment, by the 3,4,5-dimethylthiazol- 2-yl-2-5-diphenyltetrazolium bromide (MTT) method. This method evaluates the activity of a mitochondrial

112 enzyme, which is an index of cell viability. ~~The extracts did not show cytotoxicity at each~~

113 ~~concentration tested.~~

114 *2.4. Cell culture and IL-8 release measurement*

115 AGS cells were grown at 37 °C in DMEM F12 supplemented with 100 units penicillin per mL,

116 100 mg streptomycin per mL, 2 mM L-glutamine, and 10% heat-inactivated FBS (Euroclone S.p.A,

117 Pero, Italy), under a humidified atmosphere containing 5% CO2.

118 Cells were grown in 24-well plates for 48 h (30 000 cells per well) before the cytokine treatment.

119 The IL-8 secretion, induced by TNFα at 10 ng/mL, was tested after 6 h treatment in the presence of

120 un-digested or digested extracts (0,1–100 μg/mL). Curcumin (10 μM) was used as the reference

121 inhibitor of IL-8 secretion (80% inhibition). IL-8 was quantified using a Human Interleukin-8 ELISA

122 Development Kit as described below. Briefly, Corning 96 well EIA/RIA plates from Sigma- Aldrich

123 (Milan, Italy) were coated with the antibody provided in the ELISA Kit (Peprotech Inc., London, UK)

124 overnight at 4 °C. After blocking the reaction, 200 μl of samples in duplicate were transferred into

125 wells at room temperature for 1 h. The amount of IL-8 in the samples was detected by spectroscopy

126 (signal read: 450 nm, 0.1 s) by the use of biotinylated and streptavidin–HRP conjugate antibodies,

127 evaluating the 3,3′,5,5′-tetramethylbenzidine (TMB) substrate reaction. Quantification of IL-8 was

128 done using an optimized standard curve supplied with the ELISA Kit (8.0–1000.0 pg/mL).

129 *2.5. NF-κB driven transcription*

130 To evaluate the NF-κB driven transcription, AGS cells were plated in 24-well plates (30,000 cells

131 per well). After 48 h, cells were transiently transfected by the calcium-phosphate method with the

132 reporter plasmid (NF-κB-LUC, 50 ng/well) containing the luciferase gene under control of three κB

133 responsive elements. The plasmid NF-κB-LUC was a gift of Dr. N. Marx (Department of Internal

134 medicine-Cardiology, University of Ulm, Ulm, Germany). After 16 h, the cells were treated with the

135 stimulus (TNFα 10 ng/mL) and the extract for 6 h. Curcumin (10 μM) was used as the reference

136 inhibitor. At the end of this time, cells were harvested and the luciferase assay was performed using

137 the Britelite™ Plus reagent (PerkinElmer Inc., Walthman, MA, USA) according to the manufacturer's
138 instructions. Data were expressed considering 100% of the luciferase activity related to the cytokine-
139 induced promoter activity.

140 *2.6. Total Phenol Content Assay*

141 Total polyphenol content was determined according to Folin–Ciocalteu's method, as reported
142 by Singleton and Rossi [32]. Freeze-dried samples (50 mg) were solubilized in 1 mL of a 50:50
143 water:methanol solution. Aliquots of 300 μL from different samples were mixed in test tubes with 1.5
144 mL of Folin–Ciocalteu's reagent diluted 10 times, and 1.2 mL of 7.5% (w/v) sodium carbonate. After
145 30 min, the absorbance was measured at 765 nm in a UV-visible spectrophotometer (Varian Cary 50
146 SCAN, Palo Alto, CA, USA). The polyphenol content in samples was calculated using a standard
147 curve of Gallic acid. Results were expressed as equivalents of Gallic acid in mg/g.

148 *2.7. In vitro gastric digestion*

149 According to a well-established protocol, the gastric digestion was simulated using an in vitro
150 approach previously described [33]. Briefly, the extracts (100 mg) were incubated for 5 minutes at 37
151 °C with 6 mL saliva juice, then 12 mL gastric juice were added to the suspension and the sample was
152 incubated for 2 hours at 37 °C. At the end of the incubation, the digested sample was centrifuged for
153 5 minutes at 3000g and the supernatant frozen and lyophilized. All the samples were then stored at
154 −20 °C until use for biological assays.

155 *2.8. Evaluation of Thermal Stability*

156 Heating treatment was performed directly on 10 mg of dried extracts, placed in pyrex vials,
157 using a stove (Tecnovetro s.r.l., Monza, Italy) at different temperatures. The extracts were placed in
158 the stove once the selected temperature was reached. The temperature was additionally controlled
159 by a second thermometer during the incubation time. The heating treatment at 50°C reflected the
160 temperature reached by the fruit during the industrial drying process, while 100°C was selected as

161 boiling temperature, the same used for boiled chestnut preparation. After heating, 1-2 milligrams of
162 the extract were weighted and solved in a mix of water and DMSO (3:1). The highest heating time
163 (6h) at 100°C led to carbonized insoluble particles, thus the extracts were centrifuged after the
164 addition of the solvent to remove insoluble residues.

165 *2.9. UPLC-MS/MS method for multiple classes of phenolics*

166 Phenolic compounds were determined according to a previous method [34]. Briefly, an aliquot
167 of the extract was filtered 0.22 μm in a HPLC vial. Chromatographic analysis was performed using a
168 Waters Acquity UPLC system (Milford, USA) with a Waters Acquity HSS T3 column (100 mmx2.1
169 mm; 1.8 μm). The flow was 0.4 mL/min and the gradient profile was 5% B for the initial condition;
170 from 0 to 3 min linear gradient to 20% B; from 3 to 4.3 min, isocratic 20% B; from 4.3 to 9 linear gradient
171 to 45% B; from 9 to 11 min, linear gradient to 100% B; from 11 to 13 min wash at 100% B and then
172 from 13.01 to 15 min back to the initial conditions (B: acetonitrile containing 0.1% formic acid; A:
173 water containing 0.1% formic acid). Mass spectrometry detection was performed on a Waters Xevo
174 triple-quadrupole mass spectrometer detector (Milford, USA) with an electrospray (ESI) source [34].
175 A total of 24 polyphenols were identified among the samples and proper calibration curves were
176 obtained for each individual compound for precise quantification.

177 *2.10. Analysis of proanthocyanidins*

178 The assay to determine the quantity of proanthocyanidins was based on their transformation
179 into anthocyanidins, in a warm, acid environment [35]. 0.5 mL of the initial extract and 2.5 mL of
180 MeOH were collected in a 50 mL flask, shielded from light with aluminum foil, containing 9.5 mL
181 absolute EtOH. 12.5 mL of $FeSO_4$ in concentrated HCl (300 mg/L) were added to the mixture and
182 then immediately placed in a boiling water bath and refluxed for 50 min. After 10 min at room
183 temperature, the spectrum from 380 to 700 nm was recorded in a 10 mm cell, against a blank (water).
184 The tangent from the minimum (450 nm) was drawn, and the absorbance between the maximum (550
185 nm) and the tangent was measured. To subtract natural anthocyanins present in the sample, which

can interfere with the assay, 0.5 mL of the extract was prepared under the same conditions, however, in this case the reaction was carried out in an ice bath and the absorbance obtained was then subtracted to obtain the net absorbance value. The proanthocyanidins concentration (mg/g) can conventionally be expressed as the cyanidin formed. Further information is available in the literature [35-37].

2.11. Analysis of index of vanillin

The catechins and proanthocyanidins reactive to vanillin were analyzed according to the optimized and controlled vanillin-HCl method of Broadhurst and Jones [37, 38], following the conditions described by Di Stefano et al. [39]. 0.5 mL of the initial extract and 0.5 mL of MeOH were collected in a 50 mL flask, shielded from light with aluminum foil, 6 mL of vanillin (4% in methanol) were added in the flask and 3 mL of HCl. To subtract the natural interference, 0.5 mL of the extract was prepared under the same conditions, was used 6 mL of pure methanol instead of vanillin solution. The absorbance was measured at 500 nm in a 10 mm cell, against a blank reaction. Concentrations were calculated as (+)-catechin (mg/g).

2.12. HPLC analysis of Procyanidins

The PA subunit composition, percentage of galloylation (%G), percentage of prodelphinidins (%P), and mean degree of polymerisation (mDP), were determined after acid-catalysis in the presence of excess phloroglucinol (phloroglucinolysis) [40]. One hundred microliters of the sample were added to 900 μL of methanol and water (50/50 v/ v), filtered, and injected into the LC-MS system. One hundred microliters of sample were added to 100 μL of phloroglucinol reagent at 50 °C for 30 min and then combined with 1 mL of sodium acetate to stop the reaction. The samples were filtered and immediately analysed.

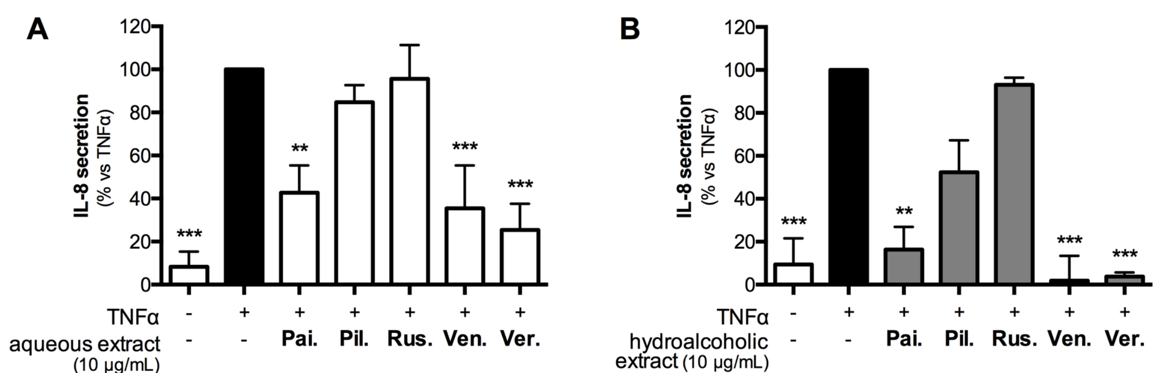
2.13. Statistical Analysis

All data are expressed as mean ± s.d.; data were analyzed by ~~unpaired~~ one-way analysis of variance (ANOVA) followed by Bonferroni as post-hoc test. Statistical analyses were done using GraphPad Prism 5.0 software (GraphPad Software Inc., San Diego, CA, USA). $p < 0.05$ was considered statistically significant. IC_{50} was calculated using GraphPad Prism 5.00 software.

3. Results

3.1. Anti-inflammatory effects of chestnut extracts in Human Gastric Epithelial (AGS) Cells

The first step in the bio-guided approach used in the present study was the preparation of aqueous and hydroalcoholic extracts from freshly collected whole chestnut fruits from five varieties of *Castanea sativa* Mill. The yields of the extracts are reported in the supplementary materials (Table S1). None of the tested extracts displayed cytotoxic effects on AGS cells, evaluated by the MTT test (data not shown). We then assayed the potential anti-gastritis properties of chestnut fruit extracts (10 μg/ml) by measuring the amount of IL-8 released by AGS cells treated with TNFα for 6 h. As shown in figure 1, only the extracts from Paié, Venégon, and Verdésa significantly prevented IL-8 release, and in all three cases the hydroalcoholic extracts exhibited higher inhibitory effect (fig. 1, panel B vs panel A).



C

Aqueous extracts	Total phenols (GAE) mg/g ± s.d.	Hydroalcoholic extracts	Total phenols (GAE) mg/g ± s.d.
Paié	32.38 ± 2.71	Paié	37.10 ± 7.66
Piliscé	18.40 ± 0.61	Piliscé	17.43 ± 3.15
Russirö	13.50 ± 0.92	Russirö	7.80 ± 3.46

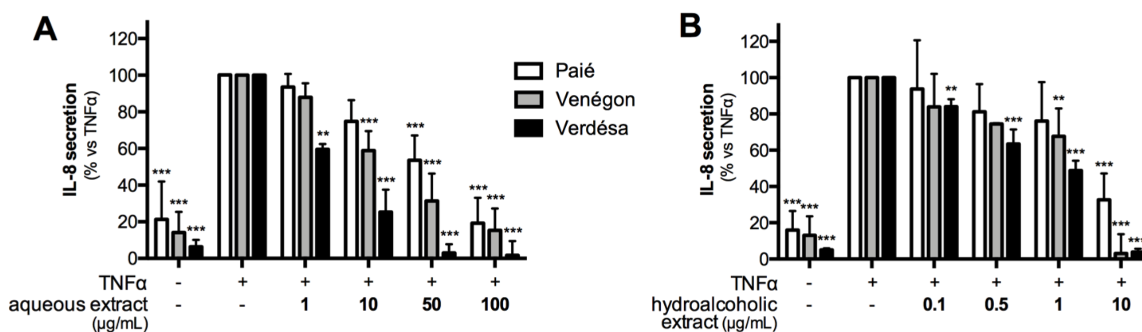
Venégon	45.00 ± 6.61	Venégon	42.80 ± 8.25
Verdésa	44.07 ± 2.38	Verdésa	96.67 ± 8.13

Paié (Pai); Piliscé (Pil); Russirö (Rus); Venégon (Ven.); Verdésa (Ver.). Total phenols results are expressed as mg gallic acid equivalents (GAE)/g extract and are the mean ± standard deviations (s.d.)

Figure 1. Effect of aqueous (A) and hydroalcoholic (B) extracts (10 μg/mL) from chestnuts of 5 different varieties on IL-8 secretion in TNFα-treated AGS cells. Amount of total phenols measured in each extract (C).

The total phenol content in the extracts ranged between 7.80 and 96.67 mg, expressed as gallic acid equivalents (GAE)/g of extract. The highest content was found in Verdésa and Venégon extracts, both aqueous and hydroalcoholic, as shown in Figure 1, panel C. In general, the anti-inflammatory activity paralleled the total phenol content. Notably, in the case of Verdésa the use of the hydroalcoholic mixture was more efficient in extracting the phenolic fraction (96.67 vs 44.07 mg/g), thus suggesting a variety-specific composition of phenolic compounds exhibiting different physico-chemical properties.

The extracts from the most active varieties were further investigated in concentration-response experiments ranging from 0.5 to 100 μg/mL for the aqueous extracts (Figure 2A) and from 0.1 to 10 μg/mL for the hydroalcoholic extracts (Figure 2B).



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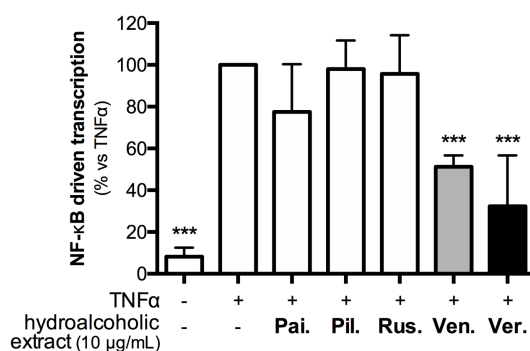
IC50 μg/mL ± s.d.	Aqueous extracts	Hydroalcoholic extracts
Paié	21.01 ± 7.09	1.85 ± 1.34
Venégon	10.22 ± 2.54	1.5 ± 0.52
Verdésa	1.44 ± 0.32	0.75 ± 0.09

244 **Figure 2.** Release of IL-8 by AGS cells treated with TNFα in the presence of aqueous (A) and
245 hydroalcoholic (B) extracts from chestnut varieties.

246 All the aqueous and hydroalcoholic extracts inhibited the release of IL-8 in a concentration-
247 dependent manner and, as expected, the hydroalcoholic extracts showed IC_{50s} between 2 and 11 times
248 lower compared to the corresponding aqueous counterparts (fig. 2C). Based on these results, the
249 hydroalcoholic extracts were selected for further investigations.

250 Since TNFα-induced expression of IL-8 depends on the NF-κB activation, we assayed the ability
251 of hydroalcoholic extracts to modulate NF-κB driven transcription. AGS cells were transiently
252 transfected with the NF-κB-luc reporter plasmid and treated for 6 h with the extracts at 10 μg/mL in
253 the presence of TNFα (10 ng/mL). The inhibitory effects of the extracts were statistically significant
254 only for the varieties Venégon and Verdésa. Furthermore, while the TNFα-induced release of IL-8
255 was completely prevented by 10 μg/mL Venégon and Verdésa hydroalcoholic extracts (fig. 1B), at the
256 same concentration the NF-κB-driven transcription was inhibited only by 50-60%. Nevertheless, these
257 results confirm that the NF-κB pathway is a key molecular target of chestnut bioactive compounds.



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259 **Figure 3.** Effect hydroalcoholic extracts (10 μg/mL) from chestnuts of 5 different varieties on NF-κB
260 driven transcription in TNFα-treated AGS cells.

261 Since in some cases, plant extracts, as single treatment, may induce an inflammatory
262 response we evaluated the release of IL-8 and NF-κB-driven transcription in AGS cells treated
263 with 10 μg/ml hydroalcoholic extract from the five chestnut varieties in the absence of TNFα. We

264 found no change of IL-8 secretion nor of promoter activity upon 6 h treatment with the five
265 extracts, thus excluding inflammatory effects in untreated cells (data not shown).

266 To get insights into the chemical entities contributing to the biological activity, we performed
267 a detailed phytochemical characterization of the hydroalcoholic extracts from the five chestnut
268 varieties, by UPLC-MS/MS. As reported in Table 1, we identified different classes of compounds,
269 including condensed tannins (e.g. proanthocyanidins), flavonoids (e.g. catechins), stilbenes (e.g.
270 resveratrol), and phenolic acids (e.g. gallic and ellagic acids). The most active varieties (Paié,
271 Venégon, Verdésa) contained significant levels (> 20 mg/g of extract) of high molecular weight
272 proanthocyanidins, with a mean degree of polymerization (mDP) ranging between 3.2 (Venégon)
273 to 6.7 (Piliscé). In contrast, these compounds were undetectable, in the least active variety,
274 Russirò, thus suggesting that this class of molecules is a major contributor to the anti-
275 inflammatory activity shown above. Considering the extraction efficiency, proanthocyanidins
276 ranged from 0 to 1.75 mg/g of the whole fruit (Table 1, last bottom line).

		LOQ mg	**Paié**	**Piliscé**	**Russirö**	**Venégon**	**Verdésa**
Gallic acid	mg/g	0,01	1.40	0.21	0.17	1.34	0.59
Ellagic acid	mg/g	0,10	3.71	0.60	0.41	5.43	1.32
Vanillin reaction (+) catechin	mg/g	0,1	6.3	4.9	3.8	12.6	62.5
Proanthocyanidin B.S. (HMWP)	mg/g	2,0	10.1	4.8	n.d.	26.7	147.1
Flavanol monomers	µg/g		0.1	0.0	0.0	0.1	0.5
Procyanidins dimers	µg/g		0.0	0.0	0.0	0.0	0.1
Procyanidins oligomers	µg/g		2.4	0.7	0.5	3.3	13.3
mDP	%		3.8	6.7	4.6	3.2	5.2
p-hydroxybenzoic acid	µg/g	0,50	24.44	6.04	3.19	38.63	5.53
Vanillic acid	µg/g	0,10	32.82	5.13	2.97	30.73	1.01
Caffeic acid	µg/g	0,02	3.79	0.03	n.d.	5.79	n.d.
Ferulic acid	µg/g	0,01	64.32	2.76	2.24	95.65	1.43
Sinapic acid	µg/g	2,00	213.43	n.d.	3.37	81.87	28.03
t-coutaric acid	µg/g	0,05	8.09	12.36	15.66	6.43	1.01
t-resveratrol	µg/g	2,00	12.98	3.18	2.42	14.49	9.91
t-piceide	µg/g	1,00	9.09	1.59	1.44	7.29	8.80
Phlorizin	µg/g	0,10	n.d.	0.57	1.38	n.d.	25.45
Luteolin	µg/g	0,20	1.28	n.d.	n.d.	0.58	n.d.
Naringenin	µg/g	0,20	16.57	0.99	n.d.	17.20	1.63
Catechin	µg/g	5,00	92.50	12.38	6.02	306.05	272.39

Gallocatechin	μg/g	100,00	549.40	121.10	n.d.	611.47	493.53
Procyanidin B1	μg/g	20,00	n.d.	n.d.	n.d.	n.d.	89.43
Procyanidin B3 (as B1)	μg/g	20,00	n.d.	n.d.	n.d.	n.d.	86.51
Taxifolin	μg/g	0,50	n.d.	n.d.	n.d.	n.d.	0.80
Quercetin-3-Rha	μg/g	0,20	n.d.	n.d.	n.d.	0.31	n.d.
Kaempferol-3-Glc	μg/g	0,20	n.d.	n.d.	n.d.	n.d.	n.d.
Isorhamnetin-3-Glc	μg/g	0,10	1.92	0.49	0.30	3.49	1.25
Isorhamnetin-3-rutinoside	μg/g	0,20	n.d.	n.d.	n.d.	n.d.	n.d.
Quercetin-3-glucuronide	μg/g	0,20	0.64	n.d.	n.d.	0.35	n.d.
Quercetin-3-Glc + quercetin-3-Gal	μg/g	0,10	0.52	n.d.	n.d.	2.09	0.10
Proanthocyanidins in the whole fruit	mg/g	2,0	0.13	0.04	n.d.	0.21	1.75

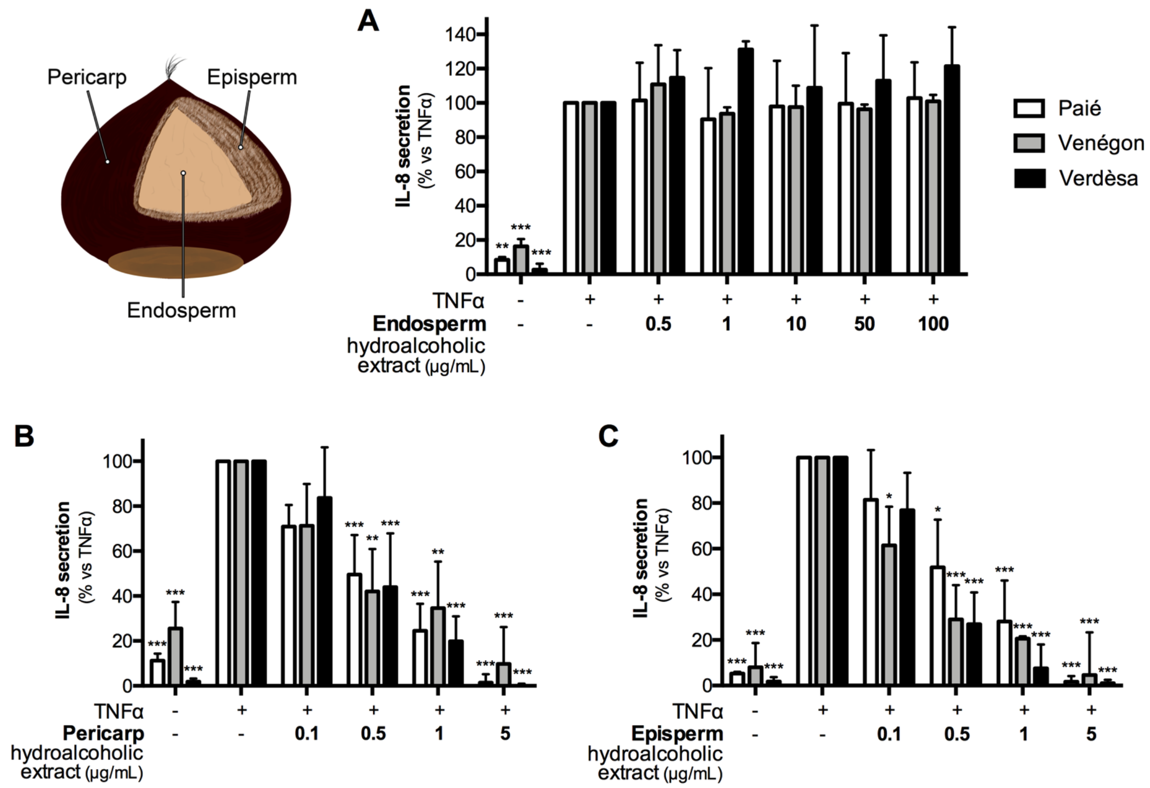
277
278 **Table 1.** Phytochemical
279
280 characterization of the hydroalcoholic extracts from the five chestnut varieties

281 In summary, we found that chestnuts contain bioactive compounds that inhibit the TNFα-
282 induced secretion of IL-8 by gastric cells, by interfering, at least in part, with the NF-κB pathway.
283 The content in bioactive compounds with anti-inflammatory properties varied among the five
284 varieties and the hydroalcoholic mixture resulted the most efficient solvent to extract active
285 molecules. The ability to inhibit IL-8 secretion correlated with the amount of proanthocyanidins
286 in the tested extracts.

287 *3.2. Contribution of the fruit parts to the biological activity*

288 To investigate the contribution of the various parts of the fruit to the biological activity,
289 hydroalcoholic extracts from the edible (endosperm) and not edible (pericarp and episperm) parts
290 were prepared separately, as described in section 2.2. In the biological assays, we tested the varieties
291 that resulted more active, i.e. Paié, Venégon and Verdésa (fig. 1 and 2).

292 The extracts obtained from endosperm, the edible part, were not active on IL-8 release till the
293 maximum concentration tested (100 μg/mL) (Figure 4A). On the contrary, the hydroalcoholic extracts
294 from pericarp and episperm showed a concentration dependent inhibitory activity (Figure 4B and
295 4C, respectively).

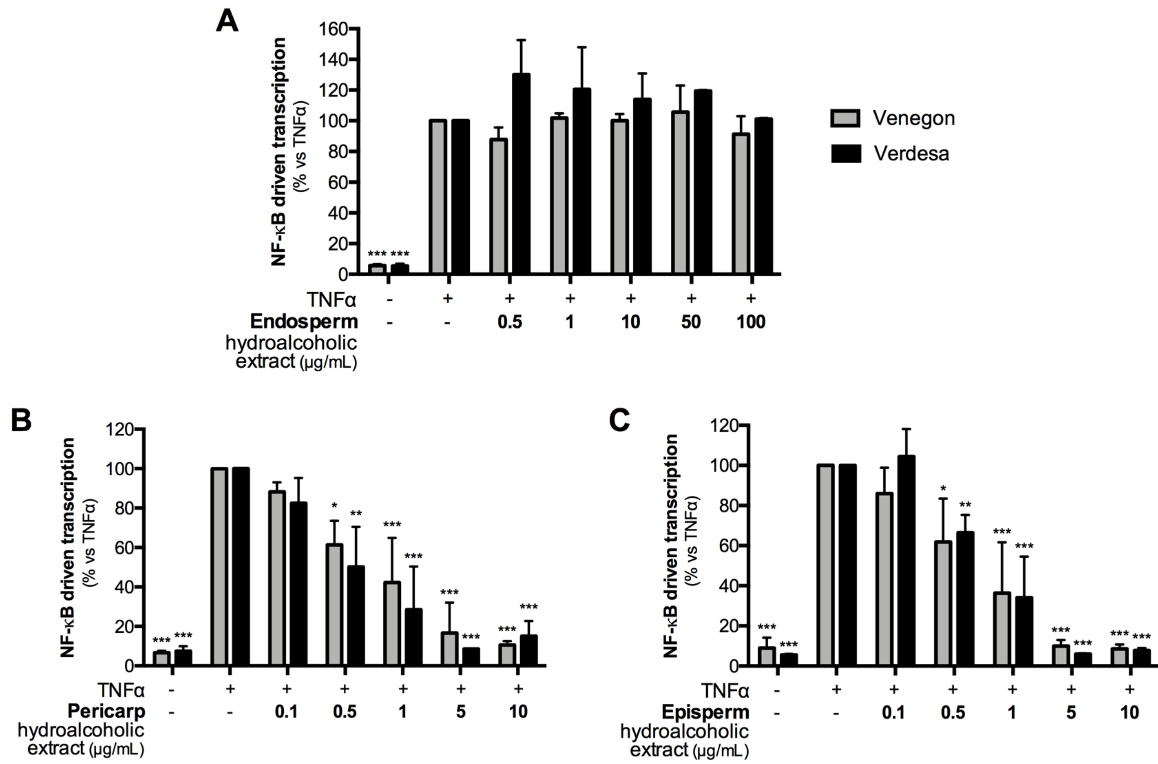


D

IC_{50} µg/mL ± s.d.	Pericarp	Episperm
Paié	0.28 ± 0.10	0.42 ± 0.12
Venégon	0.15 ± 0.08	0.14 ± 0.04
Verdésa	0.37 ± 0.08	0.22 ± 0.04

Figure 4. Hydroalcoholic extracts from pericarp (B) and episperm (C) inhibit IL-8 secretion in TNF*α*-treated AGS cells, while extract from endosperm (A) is inactive at the concentrations tested

The two varieties active on NF-κB signaling, Venégon and Verdésa (fig. 3), were further investigated; the corresponding hydroalcoholic extracts, either from pericarp (Figure 5B) and episperm (Figure 5C), impaired the NF-κB driven transcription challenged by TNF*α* in a concentration dependent manner. The hydroalcoholic extracts from endosperm displayed no activity at any of the tested concentrations (Figure 5A).



307

308 D

IC_{50} µg/mL ± s.d.	Pericarp	Episperm
Venégon	1.97 ± 0.94	1.5 ± 1.15
Verdésa	1.1 ± 0.81	2.04 ± 1.12

309

310

311 **Figure 5.** Effect of hydroalcoholic extracts (10 µg/mL) of the fruit parts from Venégon and Verdésa

312 varietes on NF-κB driven transcription in TNFα-treated AGS cells

		LOQ mg	Paié			Piliscé			Russirö			Venégon			Verdésa		
			End.	Epi.	Per.	End.	Epi.	Per.	End.	Epi.	Per.	End.	Epi.	Per.	End.	Epi.	Per.
Gallic acid	mg/g	0,01	n.d.	3.42	1.98	0.03	1.38	0.89	0.01	1.04	0.74	n.d.	2.65	1.74	0.09	0.49	0.82
Ellagic acid	mg/g	0,10	1.17	1.66	18.19	0.79	4.24	20.97	0.24	4.27	23.61	0.53	3.73	15.54	0.52	1.03	4.97
Vanillin reaction (+) catechin	mg/g	0,1	6.4	387.5	90.4	9.3	114.7	45.9	3.5	7.9	15.8	5.5	303.8	50.6	0.1	676.0	281.9
Proanthocyanidin B.S. (HMWP)	mg/g	2,0	n.d.	687.3	145.1	n.d.	235.7	76.6	n.d.	4.2	25.6	n.d.	651.4	150.2	n.d.	921.0	378.9
Flavanol monomers	µg/g		0.0	1.7	0.9	0.0	0.6	0.3	0.0	0.1	0.1	0.0	1.6	0.6	0.0	1.3	2.6
Procyanidins dimers	µg/g		0.0	0.4	0.2	0.0	0.0	0.3	0.0	0.0	0.0	0.0	0.5	0.6	0.0	0.2	3.5
Procyanidins oligomers	µg/g		0.7	11.1	211.1	0.7	53.1	7.5	0.1	0.7	2.2	0.7	13.4	188.6	0.1	352.4	112.8
mDP	%		1.0	2.3	11.8	1.3	9.1	3.3	1.0	1.7	1.8	1.3	2.9	8.2	1.5	13.2	5.0
p-hydroxybenzoic acid	µg/g	0,50	14.39	38.13	85.80	11.98	29.52	37.76	13.13	69.38	60.82	33.77	30.61	62.71	0.80	3.97	12.22
Vanillic acid	µg/g	0,10	37.95	25.39	60.54	4.55	21.11	22.19	2.57	7.54	11.35	21.57	16.29	45.27	0.92	0.83	3.98
Caffeic acid	µg/g	0,02	1.08	2.48	6.47	0.05	0.18	0.07	n.d.	n.d.	n.d.	0.76	9.56	1.83	0.58	0.02	n.d.
Ferulic acid	µg/g	0,01	25.08	11.76	2.59	1.56	2.07	0.27	0.01	0.25	0.35	21.52	47.47	11.14	8.43	1.85	1.58
Sinapic acid	µg/g	2,00	61.86	127.46	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	56.90	143.20	n.d.	53.43	n.d.	n.d.
t-coutaric acid	µg/g	0,05	41.36	37.42	3.65	5.19	n.d.	n.d.	5.50	1.41	n.d.	47.51	26.78	37.79	8.05	6.65	41.60
t-resveratrol	µg/g	2,00	n.d.	89.37	46.02	n.d.	16.60	12.84	n.d.	n.d.	5.03	n.d.	38.17	30.54	n.d.	36.94	48.50
t-piceide	µg/g	1,00	5.77	25.80	20.82	n.d.	8.02	4.86	n.d.	2.33	3.02	7.38	21.05	16.96	n.d.	3.03	8.87
Phlorizin	µg/g	0,10	n.d.	17.91	34.69	n.d.	1.23	7.78	n.d.	0.12	0.55	n.d.	16.09	53.13	0.52	22.48	13.88
Luteolin	µg/g	0,20	n.d.	1.34	11.00	n.d.	0.28	5.79	n.d.	n.d.	2.26	0.28	0.40	7.44	n.d.	0.35	9.31
Naringenin	µg/g	0,20	5.75	74.68	47.06	n.d.	3.29	5.19	n.d.	n.d.	0.55	5.14	39.98	54.54	1.09	14.09	20.62
Catechin	µg/g	5,00	n.d.	1533.04	2231.3	n.d.	170.38	213.67	n.d.	11.73	154.43	22.95	1974.71	1648.55	n.d.	286.76	1942.00
Gallocatechin	µg/g	100,00	n.d.	1642.02	613.70	n.d.	547.02	356.55	141.87	216.17	n.d.	n.d.	1228.70	571.09	n.d.	611.34	486.20
Procyanidin B1	µg/g	20,00	n.d.	249.47	1056.2	n.d.	n.d.	104.56	n.d.	n.d.	59.62	n.d.	507.95	941.71	n.d.	62.12	827.09
Procyanidin B3 (as B1)	µg/g	20,00	n.d.	287.11	336.46	n.d.	n.d.	81.10	n.d.	n.d.	52.88	n.d.	1193.32	319.10	n.d.	69.82	378.94
Taxifolin	µg/g	0,50	n.d.	18.44	18.08	n.d.	n.d.	2.10	n.d.	n.d.	1.02	n.d.	19.67	17.71	n.d.	1.18	13.17
Quercetin-3-Rha	µg/g	0,20	n.d.	0.75	3.02	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.86	7.48	n.d.	n.d.	4.50
Kaempferol-3-Glc	µg/g	0,20	n.d.	n.d.	2.97	n.d.	n.d.	1.03	n.d.	n.d.	n.d.	n.d.	n.d.	1.40	0.43	n.d.	0.47
Isorhamnetin-3-Glc	µg/g	0,10	n.d.	3.94	102.86	n.d.	n.d.	8.48	n.d.	n.d.	0.97	n.d.	1.94	135.92	n.d.	0.20	47.24
Isorhamnetin-3-rutinoside	µg/g	0,20	n.d.	0.97	5.82	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1.37	4.48	n.d.	n.d.	0.83
Quercetin-3-glucuronide	µg/g	0,20	15.03	0.69	41.21	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.63	0.28	5.58	n.d.	n.d.	0.63
Quercetin-3-Glc + quercetin-3-Gal	µg/g	0,10	5.25	1.47	23.77	n.d.	0.14	0.53	n.d.	n.d.	0.69	0.43	0.75	10.55	0.29	0.20	4.42

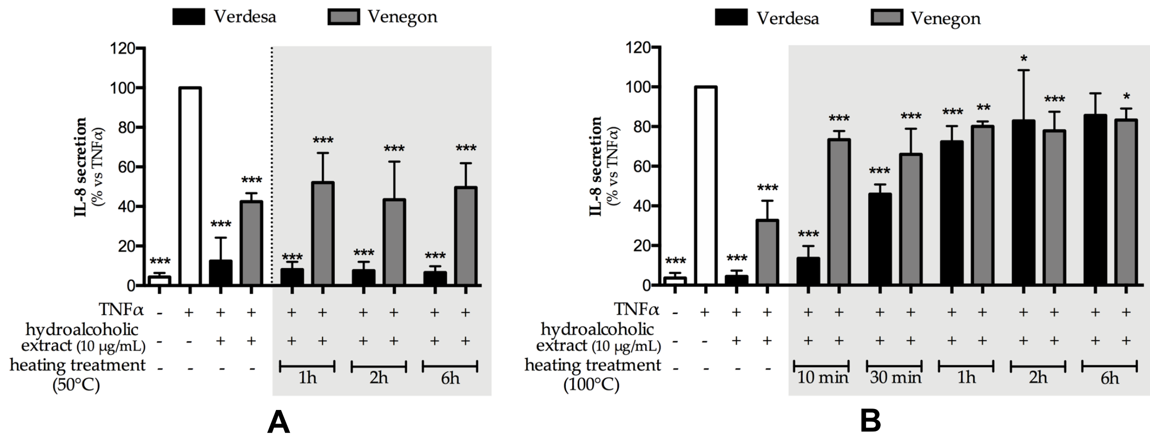
Table 2. Phytochemical characterization of the hydroalcoholic extracts of the fruit parts from the five chestnut varieties

327 The finding that none of the extracts from the edible part (endosperm) of the most active
328 varieties displayed biological activity (fig. 4A and 5A) prompted us to investigate in more detail
329 the phytochemical composition of the various extracts. As reported in Table 2, high molecular
330 weight proanthocyanidins, the putative bioactive fraction, were enriched (>650 mg/g) in the
331 extracts obtained from the episperm of Paiè, Venégon and Verdésa. The same applies to the
332 extracts prepared from the pericarp, with concentration ranging from 145.1 mg/g (Paié) to 378.9
333 mg/g (Verdésa). The variety Verdesa resulted the richest source of high molecular weight
334 proanthocyanidins, which accounted to about 90% of the episperm extract. Russirò, the variety
335 exhibiting the lowest anti-inflammatory activity (fig. 1B), resulted almost devoid of
336 proantocyanidins in any part. The anti-inflammatory activity resulted unrelated to the mean
337 degree of polymerization (mDP) of proanthocyanydins. In fact, for example, the extracts from
338 both pericarp and episperm of Venégon fruits displayed IC_{50} of about 0.15 μg/mL (Fig. 4D), while
339 the mDP accounted to 8.2 and 2.9, respectively.

340 The results shown above indicate that the biological activity is associated to the not edible
341 parts of chestnut (pericarp and endosperm) in all the tested varieties. The phytochemical analysis
342 confirmed that the hydroalcoholic extracts exhibiting the highest anti-inflammatory activity were
343 richest in high molecular weight proanthocyanidins; the observation corroborates the hypothesis
344 that these compounds confer to chestnut extracts the ability to prevent IL-8 secretion.

345 *3.3. Effect of extrinsic variables on the biological activity of chestnut varieties*

346 Chestnuts are widely used in food industry for sweets and flour production, through procedures
347 that require heating. Therefore, we evaluated the anti-inflammatory property of hydroalcoholic
348 extracts from Verdésa and Venégon chestnuts upon exposure to mild (50°C) and high (100°C)
349 temperature, as described in section 2.2. As shown in Figure 6A, when heated at 50°C up to 6 h, both
350 extracts maintained the inhibitory activity on IL-8 secretion. However, both extracts exhibited
351 reduced activity upon exposure at 100°C in a time dependent manner; of note, the inhibitory effect
352 was almost completely lost after 2 h of incubation at 100°C (Figure 6B).



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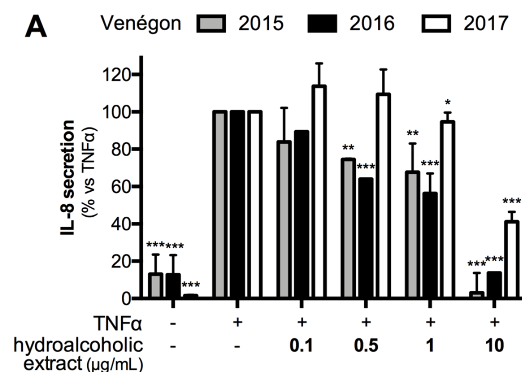
354 **Figure 6.** Heating treatment of dried hydroalcoholic extracts from chestnuts varieties: (A) the extracts
355 inhibit IL-8 secretion in TNFα-treated AGS cells after heating at 50 °C for 6h. (B) time and temperature
356 dependent loss of IL-8 inhibition in TNFα-treated AGS cells during heating at 100 °C

357 The results shown so far refer to extracts prepared from fruits harvested in the year 2015. It is
358 known that the quali-quantitative composition in bioactive compounds of medicinal and edible
359 plants varies depending on multiple variables, including the harvest year; therefore, it is conceivable
360 that the extent of the associated biological activities may vary as well. Given the low availability of
361 fruits of the Verdésa variety, we focused on Venégon chestnuts. Hence, additional hydroalcoholic
362 extracts from the whole fruit of Venégon were prepared from chestnuts collected in 2016 and 2017.
363 The inhibitory activities (IC_{50}s) exerted by Venégon (2015) and Venégon (2016) were comparable,
364 while the extract of Venégon (2017) was less effective (Figure 7A). Although we did observe different
365 degree of activity depending on the harvest year, most likely due to the amount of bioactive
366 proanthocyanidins, the calculated IC_{50s} were in all cases below 10 μg/mL (fig. 6B).



367 ini

368 **B**

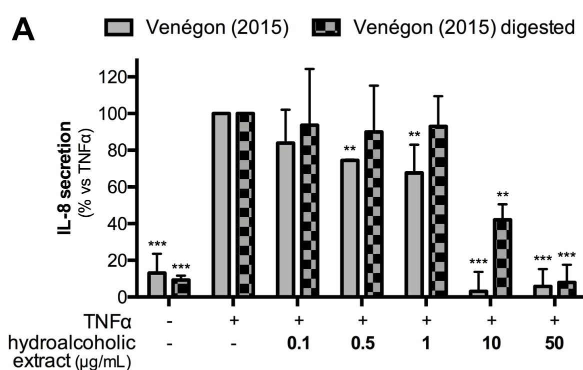
IC_{50} µg/mL ± s.d.	**2015**	**2016**	**2017**
Venégon	1.5 ± 0.52	1.34 ± 0.8	6.05 ± 0.92

369

370 **Figure 7.** Inhibition of IL-8 secretion by extracts from chestnuts harvested in different years in TNF-α-
371 treated AGS cells

372

373 A critical issue in nutraceuticals and botanicals is their fate in the gastro-intestinal tract,
374 including chemical stability and absorption, which affect the biological activities. Chemical stability
375 in the stomach environment is certainly highly relevant for extracts and compounds acting directly
376 on gastric cells. To this end we investigated whether the prevention of IL-8 secretion could be
377 maintained under the acidic conditions of the stomach and the action of digestive enzymes. The
378 hydroalcoholic extract of the whole fruit of the Venégon variety (harvest year 2015) was subjected to
379 *in vitro* simulated gastric digestion, as described in section 2.7. The simulated digestion only slightly
380 affected the biological activity of the extract (fig. 8A), increasing the IC_{50} from 1.50 ± 0.52 to 4.13 ±
381 1.83 µg/mL (± s.d.), as reported in Figure 8B.



382

383 **B**

IC_{50} µg/mL ± s.d.	**2015**	**2015 digested**
Venégon	1.5 ± 0.52	4.13 ± 1.83

384

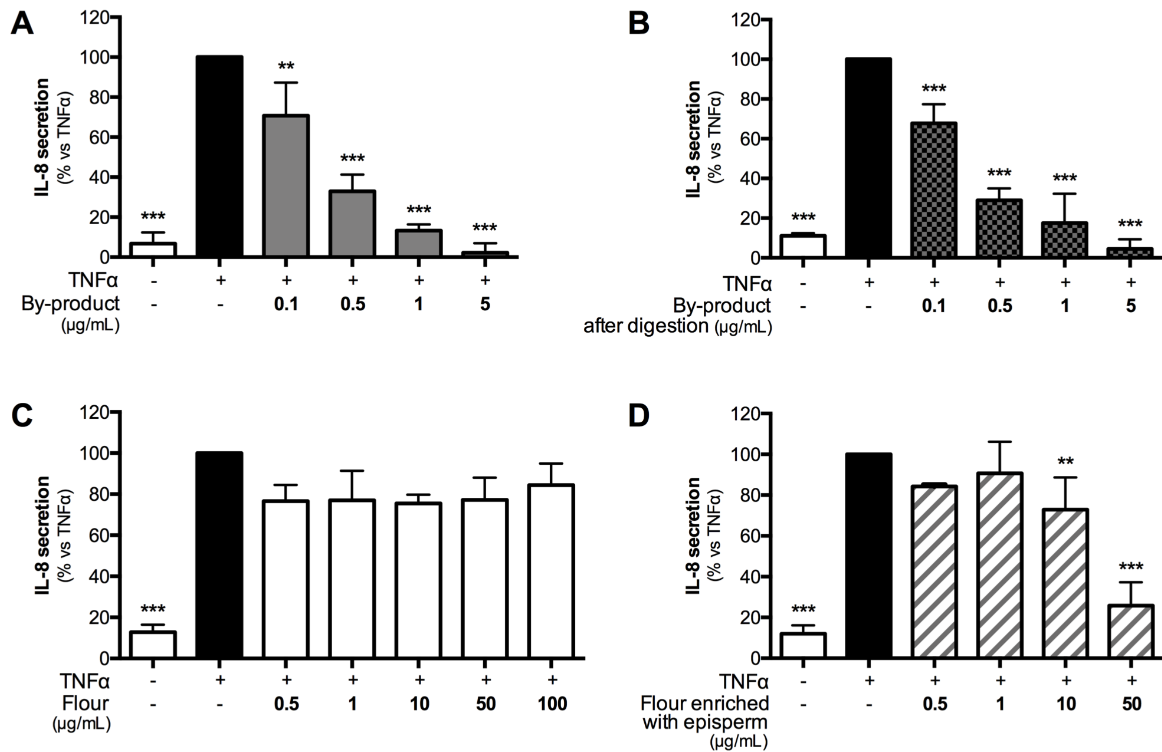
385 **Figure 8.** IL-8 secretion in TNFα-treated AGS cells in the presence of chestnut extract subjected to in
386 vitro simulated gastric digestion (A)

387 The results shown above indicate that, taking into account critical variables (thermal treatment,
388 harvest year, gastric digestive environment), Venégon chestnut resulted a valuable source of
389 bioactive compounds with potential anti-inflammatory activities at the gastric level.

390 *3.4. Biological activity of industrial chestnut flour and related by-product*

391 To verify the practical application of our results, we investigated the biological activity of
392 hydroalcoholic extracts from finished products (flour) and by-product obtained by industrial
393 processing of Venégon chestnuts (harvest year 2015). The extract prepared from by-product,
394 consisting of the not edible parts (pericarp and episperm) and resulting from mechanical peeling,
395 displayed inhibitory activity on IL-8 secretion (Figure 9A), in line with the results shown in figure 4B
396 and C; the calculated IC_{50} was 0.20 ± 0.04 μg/mL (± s.d.), highly similar to that reported in figure 4D.
397 These results indicate that the mechanical peeling procedure does not alter the potential biological
398 activity of the not edible chestnut parts. The *in vitro* gastric digestion did not affect the biological
399 activity of the extract, as shown in Figure 9B, which still exhibited an IC_{50} of 0.15 ± 0.02 μg/mL (± s.d.),
400 consistent with the findings reported in fig. 8A. On the contrary, the extract prepared from industrial
401 flour, mainly constituted of endosperm, showed absence of activity (Figure 9C), confirming the
402 results reported in figure 4A. Thus, our data suggest that chestnut-based products prepared
403 exclusively from endosperm lack anti-inflammatory activity, while industrial by-product may
404 represent a valuable source of bioactive material.

405 In the view of improving the nutraceutical properties of chestnut flour, we prepared a
406 hydroalcoholic extract starting from a mixture of flour and episperm, maintaining the ratio found in
407 the fruits (22:3, for endosperm and episperm, respectively). We used flour, and episperm from
408 Venégon fruits harvested in 2015. The extract prepared from flour enriched with episperm (final
409 concentration 12%, w:w) showed inhibitory activity with an IC_{50} of 16.35 ± 5.22 μg/mL (± s.d.), as
410 shown in Figure 9D.



411

412 **Figure 9.** Hydroalcoholic extracts from chestnuts by-products (var. Venégon) inhibit IL-8 secretion in
413 TNFα-treated AGS before (A) and after (B) a simulated gastric digestion; extract from chestnut flour
414 (C) inhibits IL-8 secretion only after enrichment with episperm (12% of final weight) (D).

415 In conclusion, we demonstrated that chestnut flour is devoid of any anti-inflammatory
416 activity while industrial by-product retains the ability to inhibit IL-8 secretion. These findings
417 provide experimental evidence in support of the potential use of chestnut-derived material for
418 the preparation of nutraceuticals and functional foods, e.g. “fortified” flour.

419 **4. Discussion**

420 *Castanea sativa* Mill. is a rich source of polyphenols due to its high tannin content, and its fruits
421 are widely used in food industry for sweets and flour production [61]. Nevertheless, a detailed
422 phytochemical profile of chestnut fruits, in relation to their biological activities, has not been
423 investigated in depth.

424 By means of a validated bio-guided approach, coupled to a thorough phytochemical analysis,
425 we were able to isolate the bioactive fraction of chestnut fruits exhibiting anti-inflammatory activity
426 in gastric cells. This approach was instrumental for the selection of the most appropriate extraction
427 system (hydroalcoholic mixture vs water), thus resulting more informative than the simple
428 measurement of the phenolic content.

429 Proanthocyanidins represent an emerging class of anti-inflammatory compounds and our study
430 further supports their pharmacological potential in the field of gastric inflammation. The detailed
431 phytochemical characterization of various chestnut varieties and fruit parts, coupled to the robust
432 biological assay, allowed us to assign the anti-inflammatory activity to this class of compounds, to
433 identify the most active varieties and the contribution of the fruit parts. Thus, chestnut fruit,
434 especially the inner and outer skins, can be included in the list of natural sources of
435 proanthocyanidins. Moreover, the amount of proanthocyanidins could represent an index to titrate
436 tannin enriched nutraceuticals.

437 Although our data clearly show that the edible part of chestnut fruits was not active, the by-
438 product resulted highly enriched in proanthocyanidins and, consequently, with significant anti-
439 inflammatory activity ($IC_{50} < 1$ μg/ml). These findings contribute to assessing the health beneficial
440 value of chestnut-based foods and nutraceuticals, and to the potential valorization of chestnut
441 processing wastes.

442 Another outcome of our study is the valorization of *Castanea sativa* Mill. varieties that combine
443 appreciated organoleptic properties with high nutraceutical value, such as the Venégon and Verdésa
444 varieties. Future efforts should be aimed at promoting preservation and cultivation of these varieties,
445 optimizing the use and manipulation of by-product, with the aim to develop chestnut-based
446 nutraceuticals and functional foods, with no negative effects on their palatability and taste. These
447 aspects will be investigated in the near future.

448 Our study also provides hints that may result useful for optimizing the processing of chestnut-
449 based products and the treatment of wastes. Elevated temperatures (>100°C) are often used to
450 facilitate the peeling procedure, to prepare chestnut-based foods (e.g. cakes, pasta, etc.), or to increase
451 the extraction efficiency in preparing chestnut-derived ingredients. Our data demonstrate that
452 prolonged exposure (>1 h) at 100°C leads to complete loss of anti-inflammatory activity while upon
453 treatment at 50°C the biological activity is fully preserved.

454 Key steps that may influence the biological activities associated to nutraceuticals–are their
455 chemical modifications that may occur during the digestive process and the absorption rate by the
456 gastro-intestinal tract. In the case of gastric inflammation, absorption may be less relevant since
457 bioactive compounds can act directly on gastric cells, but resistance to acid environment and enzymes
458 is a crucial issue. By using an *in vitro* simulated digestion system, we could demonstrate that chestnut
459 extracts maintain the ability to prevent IL-8 secretion, thus providing evidence in support of their
460 potential use *in vivo*. Moreover, taking into consideration the amount of proanthocyanidins that could
461 be added to chestnut-based foods (e.g. flour, flakes, etc.), the calculated IC_{50} reported in the present
462 study, the approximate gastric volume (30-40 mL), it is conceivable that concentrations sufficient to
463 obtain anti-inflammatory effects could be easily reached *in vivo*.

464 **5. Conclusion**

465 Overall, by combining a robust bio-guided approach with a comprehensive analysis of the tannic
466 fraction of chestnut extracts, we provided evidence for the potential use of chestnut-based
467 nutraceuticals in human gastritis. The bioactive components of chestnut fruits inhibit IL-8 secretion
468 most likely by means of multiple mechanisms, including impairment of NF-κB signaling, thus
469 confirming the importance of this pathway as a general target in the field of anti-inflammatory agents;
470 on the other hand, the discovery of additional targets would open new applications for inflammation-
471 based diseases.

The finding that the anti-inflammatory activity is maintained upon treatment in acidic conditions and with digestive enzymes, further corroborates the applicability of our observations in human gastritis. Finally, our study also offers hints useful for the valorization of specific chestnut varieties and for setting up the most appropriate conditions for the preparation of chestnut-based nutraceuticals with intact biological activities.

Conflict of interests

The authors declare no conflict of interest

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599

600 **Supplementary material**

Fruit variety and part used	Solvent for extraction	Yield (% w/w)
Paié		
Whole fruit	Water	10.3
	Water/ethanol	8
Pericarp	Water/ethanol	3.3
Episperm		16
Endosperm		9.6
Piliscé		
Whole fruit	Water	12.19
	Water/ethanol	12.97
Pericarp	Water/ethanol	3.48
Episperm		5.16
Endosperm		15.28
Russirö		
Whole fruit	Water	11.1
	Water/ethanol	11.96
Pericarp	Water/ethanol	3.48
Episperm		0.64
Endosperm		6.52
Venégon		
Whole fruit	Water	11.4
	Water/ethanol	12.6
Pericarp	Water/ethanol	6
Episperm		21
Endosperm		14.5
Verdésa		
Whole fruit	Water	16.3
	Water/ethanol	8.4
Pericarp	Water/ethanol	3.92
Episperm		27.9
Endosperm		16.5

601 Aqueous extracts (Water); hydroalcoholic extracts 50:50, ethanol:water (Water/ethanol)

602 **Table S1.** Extraction yields expressed as percentage (w/w) in respect to the starting material.