Cabo, S.; Aires, A.; Carvalho, R.; Pascual-Seva, N.; Silva, AP.; Gonçalves, B. (2021). Corylus avellana L. Husks an Underutilized Waste but a Valuable Source of Polyphenols. Waste and Biomass Valorization. 12(7):3629-3644. https://doi.org/10.1007/s12649-020-01246-4

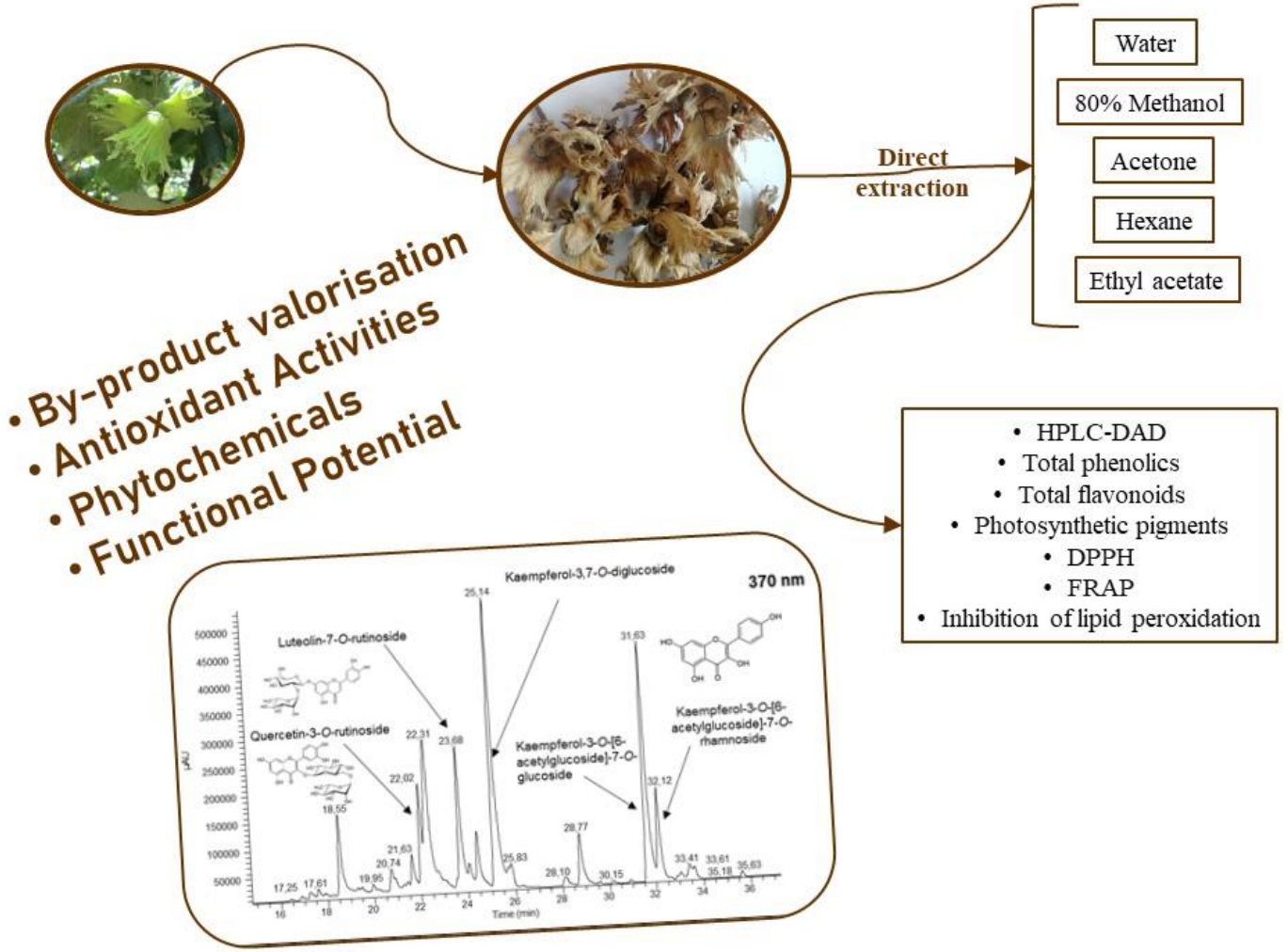
6.CORYLUS AVELLANA L. HUSKS AN UNDERUTILIZED WASTE BUT A VALUABLE SOURCE OF POLYPHENOLS

6.1. Abstract

Bioactive potential of hazelnut husks was determined as a function of their cultivar source and extraction solvent. Hazelnut husks from four hazelnut cultivars ('Butler', 'Grada de Viseu', 'Lansing' and 'Morell') were picked in a hazelnut orchard at harvest and extracted with five solvents with different polarity: water, methanol, acetone, ethyl acetate and hexane. Phenolics were identified by HPLC-DAD and antioxidant activity was determined by three complementary methods: DPPH, FRAP and inhibition of lipid peroxidation. A total of 11 phenolics were identified in studied cultivars and grouped in five main classes namely, ellagitannin (ellagic acid), benzoic acids (gallic acid, protocatechuic acid and vanillic acid), flavonols (kaempferol-3,7-*O*-diglucoside, kaempferol-3-*O*-[6-acetylglucoside]-7-*O*-glucoside, kaempferol-3-*O*-[6acetylglucoside] -7-*O*-rhamnoside and quercetin-3-*O*-rutinoside), flavone (luteolin-7-*O*-rutinoside) and flavan-3-ol (epicatechin). Cultivar and extraction solvent influenced significantly ($p<0.001$) the extraction yield. 'Grada de Viseu' husks presented the highest content of individual phenolics identified, particularly in methanol extracts whilst 'Lansing' showed the lowest levels. Similar pattern was found for antioxidant activities. Methanolic husk extracts exhibited the greatest antioxidant potentials followed by water and acetone. The valorization of hazelnuts by-products gives an important contribution for the isolation and purification of bioactive molecules that can be used for both medicinal and industrial purposes.

Keywords: Hazelnut; cultivars; husks; phenolic compounds; bioactivities solvents; extraction; biological potencial.

6.1.1. Graphical Astract



6.2. Statement of novelty

This is the first study in which hazelnut husks are valorized as by-products, since it demonstrates their richness in polyphenols with antioxidant and anti-radical activities, in a detailed mode. In addition, this study shows the potential of this neglected and underused natural resource to extract polyphenols, which can be tested in future forthcoming works as food preservative for food industry, or as anti-inflammatory, antioxidant, antibacterial, anti-cancer or cardioprotective agent for pharmaceutical and cosmetic industry. On the other hand, the results from this study can open different perspectives to deal the excess of this crop waste, including environmental friendly solutions.

6.3. Introduction

Hazelnut (*Corylus avellana* L.) belongs to the family Betulaceae, it is one of the World's major nut crops and it is one of the most nutritious nuts (Molnar et al., 2005; Lopez-Calleja et al., 2005). Hazelnut is consumed raw or roasted, being largely used in foods, particularly in bakery and confectionery products due to its flavour properties (Lopez-Calleja et al., 2005; Shahidi et al; 2007). The majority of hazelnut production is located in Asia and Black Sea countries (Bacchetta et al., 2005). However, Mediterranean countries such as Italy, Spain, and France, present considerable production yields (FAOStat, 2016). Although with low area and production yield, Portugal has favourable environmental conditions for the production of this nut. The majority of hazelnut production is consumed as dry fruit or it is used in processing industry of chocolate, jams, creams and food pastes. In addition to the hazelnut fruit production and its usage in processing industry, tones of residues such as skins, hard shells and hazelnut green leafy cover, known as husks (Shahidi et al., 2007), are every year generated without any substantial utilization besides composting or as heat source by combustion (Uzuner et al., 2014). Traditionally, hazelnut producers and industry burn these materials (Çöpür et al., 2007; Guney 2013), which raises several environmental issues, such as air and soil pollution. Therefore, environmental friendly solutions are needed to overcome this problem. Many studies have been published about the phytochemical composition of many parts of hazelnut by-products (leaves, skins and shells) (Shahidi et al., 2007; Masullo et al., 2017; Yuan et al., 2018) but few, have been published about the type and amount of phenolics present in hazelnuts green leaf covers

(Shahidi et al., 2007), and none has studied the influence of solvent polarity in both phenolic content and profile. In addition, during the last decades phenolics have been target due to its beneficial effects in human health, exhibiting antioxidant, antitumor, anticancer, antimicrobial, anti-aging, and anti-inflammatory and neuroprotective properties (Li et al., 2013; 2014). In addition, the need to replace synthetic antioxidants, which have been related to toxic and carcinogenic effects (Bouayed et al., 2010), increased the search for new and natural antioxidants, including phenolics. Exhaustively qualitative and quantitative analytical methods have been developed to obtain phenolics from natural resources including extraction, separation, detection and structure determination methods (Ignat et al., 2011). These methods usually apply freeze-drying steps to remove moisture from plant parts, blender, mill or liquid nitrogen protocols to yield fine powders, and the extraction procedure normally undergoes with solid-to-liquid extraction with moderate acidic organic solvents. Large amount of information can be found on the effect of the type of solvent on the extraction of antioxidant phenolics from different raw materials, but studies on the extraction of these compounds from hazelnuts green leaf covers are too scarce. Therefore, this study aimed (i) to evaluate the efficiency of five solvents with different polarity in the extraction of phenolics (ii) its influence on both phenolic profile and content, and (iii) to measure its bioactive potential trough *in vitro* antioxidant activity bioassays. The effectiveness of the investigated procedure is measured in terms of amount of phenolics extracted and antioxidant activity of extracts.

6.4. Material and methods

6.4.1. Experimental design and sampling

The experiment was carried with hazelnut husks collected from a hazelnut orchard in Vila Real, Portugal (lat. 41°18´N; long. 7°44´W; altitude 462 m above sea level) with adult trees in full production, spaced 3 x 4 m. Standard cultural practices such as pruning, weed and disease control were implemented regularly following the local practices. The orchard was not irrigated. The climate of the region is typically Mediterranean, characterized by warm-temperate climate with dry and hot summers, according to Koppen-Geiger climate classification, the climate of the site is Csb (Kottek et al., 2006). The air temperature (ºC) and precipitation (mm) in the hazelnut orchard were monitored at the trial by an automatic weather station (IMT280, iMETOS, Weiz, Austria) (Supplementary Table). Hazelnut husks were

obtained from four cultivars: 'Butler', 'Grada de Viseu', 'Morell' and 'Lansing' that were collected on 7th October, simultaneously at the commercial maturity stage of fruits. For each cultivar, healthy hazelnut husks were collected randomly from three trees replicate three times (n=9), at fruit harvest and transported to laboratory, and dehydrated at 40ºC in a hot chamber for 48 hours. The husks were then milled in a commercial blender into a fine dried powder.

6.4.2. Chemical reagents

2,2-diphenyl-1-picrylhydrazyl (DPPH), acetone, calcium carbonate ($CaCO_3$), ethanol, ethyl acetate, hexane, hydrochloric acid (HCl), iron (III) chloride, phosphate buffer, tiobarbituric acid (TBA), trichloroacetic acid (TCA) and trifluoroacetic acid (TFA) were purchased in Sigma Aldrich (Taufkirchen, Germany). External phenolic standards were purchased from Extrasynthese (Lyon Nord, Genay Cedex, France). All organic solvents were HPLC grade. Ultrapure water from purified system (Isopad Isomantle, Gemini BV, Pr. Beatrixlaan 301, 7312 DG Apeldoorn, Netherlands) was used.

6.4.3. Phytochemicals quantification

6.4.3.1. Extraction

The extraction method was adapted from John et al. (2018), with several modifications. 240 mg of each sample was weighed and extracted with 1 mL of five solvents with different polarity: (i) water, (ii) aqueous methanol 80%, (iii) acetone, (iv) ethyl acetate and (v) hexane. Each mixture was agitated thoroughly in a vortex and placed in a thermoblock at 70 ºC for 45 min, and then centrifuged (Centrifuge 5804 R, Eppendorf, Hamburg, Germany) at 4000 rpm for 15 min. Then, the extracts were filtered (Fisherbrand Ø 90mm) and supernatants were transferred to separate vials and residues rejected. The aqueous supernatant was dehydrated (Scanvac CoolSafe 55-4 Pro, Labogene, Lynge, Danemark) in to a dried fine powered and the supernatants from other solvents were completely dried in a rotary evaporator (40 ºC). The dried powders of each sample was then diluted in methanol (40 mg solid: 1 mL solvent) and stored in amber vials (Chromacol 2-SVWK(A)ST-CPK, ThermoScientific, Langerwche, Germany) at -20ºC until further analysis.

6.4.3.2. Total phenolic compounds (TPC)

TPC of extracts were determined by classical colorimetry method performed in 96-microplate wells (Costar 3590, Corning, NY, USA) adapted from Singleton and Rossi (1965) (Singleton and Rossi, 1965). After the extraction, 20 µL of each extract was added to each microplate well, followed by addition of 100 µL of Folin-Ciocalteu´s phenol reagent (1:10 in dd H_2O) and 80 µL of 7.5% Na_2CO_3. Then, microplates were heated at 45º C for 15 min and the absorbance values were recorded at 765 nm wavelength in a microplate reader (Multiskan™ GO Microplate Spectrophotometer, Thermo Scientific, Vantaa, Finland), against blank (all reagents except sample). The results were then expressed as mg gallic acid equivalent (GAE) g^{-1} dry weight (dw), using a calibration curve of gallic acid at different concentrations (starting from 0.0 to 1.0 mg mL^{-1}).

6.4.3.3. Total flavonoids (TFC)

TFC of extracts were determined by classic colorimetry (Dewanto et al., 2002), but performed in 96 performed wells (Costar 3590, Corning, NY, USA). 25 µL of each extract prepared previously was added to each microplate well followed by 100 µL of ultra-pure water and 10 µL of 5% $NaNO_2$. Then, the microplates were incubated at room temperature during 5 min in a dark environment. After this period, 15 µL of 10% $AlCl_3$ was added to each well and the microplates were once again incubated at room temperature in a dark environment but for 6 minutes. Then, to each well, 50 µL of NaOH 1 M followed by 50 µL of ultra-pure were added and mixed thoroughly. The absorbance values were then recorded at 510 nm in microplate reader (Multiskan™ GO Microplate Spectrophotometer, Thermo Scientific, Vantaa, Finland) against blank (all reagents and ultrapure water instead of extract). The results were expressed as mg catechin equivalent (CAE) g^{-1} dw using a calibration curve with commercial standard of catechin at different concentrations (starting from 0.0 to 1.0 mg mL^{-1}).

6.4.3.4. Photosynthetic pigments

Chlorophyll a, chlorophyll b and total carotenoids were determined by colorimetric method of Lichtenthaler and Wellburn (1983) with small modifications. 200 µL of each extract was added into a 96-wells microplate and the absorbance values were recorded in a microplate

reader (Multiskan™ GO Microplate Spectrophotometer, Thermo Scientific, Vantaa, Finland) at 470, 655 and 666 wavelengths against blank (pure solvent used in pigments extraction instead of sample). The pigment concentration in each extract was calculated from 1 cm corrected pathlength using the following formulas:

Chlorophyll a (µg mL^{-1}) = Chl a= (15.65 x Abs_{666}) – (7.94 x Abs_{655})

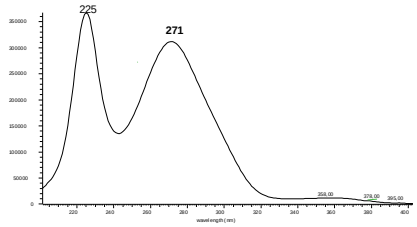
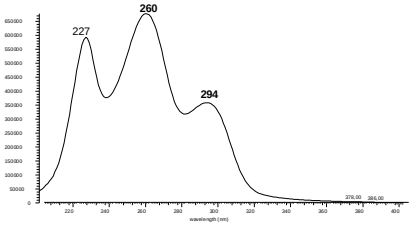
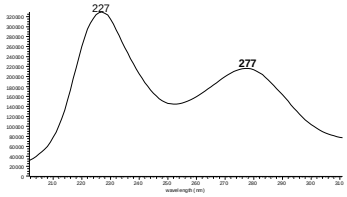
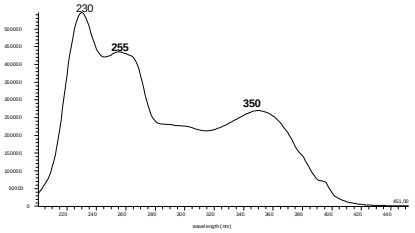
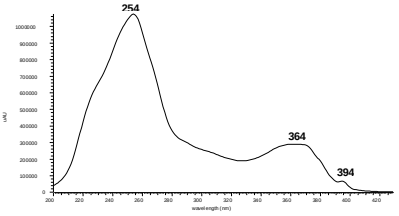
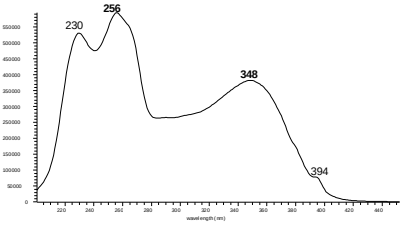
Chlorophyll b (µg mL^{-1}) = Chl b= (27.05 x Abs_{655}) – (11.21 x Abs_{666})

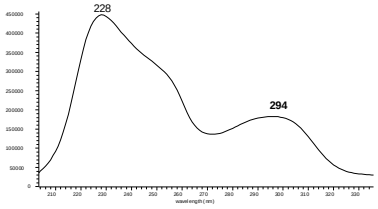
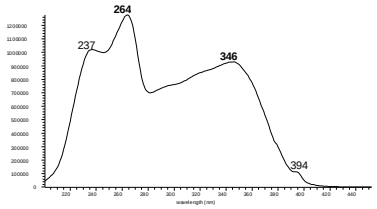
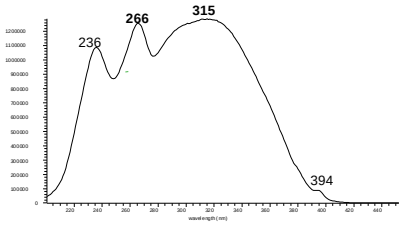
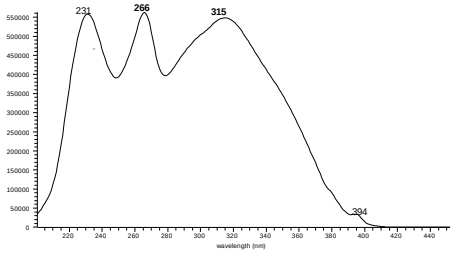
Total Carotenoids (µg mL^{-1}) = ((1000 x Abs_{470}) - (2.86 x Chl a) - (129.2 x Chl b)) / 245

6.4.3.5. Individual phenolic compounds

The phenolics present in all extracts were quantified in a HPLC-DAD system using the method of Aires et al. (2013), but with small modifications as follows: 10 µL of each extract was injected in HPLC-DAD system with an eluent composed by water with 0.1% of TFA (solvent A) and acetonitrile with 0.1% TFA (solvent B). The elution was performed with a flow rate of 1 mL min^{-1}, with a gradient starting from 0% solvent B at 0 min, 0% solvent B at 5 min, 20% solvent B at 15 min, 50% B at 30 min, 100% solvent B at 45 min, 100% solvent B at 50 min, 0% solvent B at 55 min and 0% solvent B at 60 min. The chromatograms were recorded at 254 and 280 nm for benzoic acids and flavan-3-ols, respectively, 320 nm for cinnamic acids, and 370 nm for flavonoids, with a C18 column (250 x 46 mm, 5 mm, ACE HPLC Columns, Advanced Chromatography Technologies Ltd, Abeerden, Scotland, UK). Phenolics were identified using peak retention time, UV spectra, and UV max absorbance bands (Table 6.1.) and by comparison with external commercial standards (Extrasynthese, Cedex, France). The content of each phenolic was calculated using the internal standard (naringin) method. The results were expressed as mg g^{-1} dry weight (dw).

Table 6.1. Phenolics identified in hazelnut husks and respective retention time (Rt), wavelengths of maximum absorption (λ_{max}) (by elution order) in different solvents (water, methanol, acetone, ethyl acetate and hexane).[1, 2]

Phenolics identified	Average Rt (min)	UV maximum absorption (nm)	Reference UV-DAD bands (nm)[2]	UV-DAD Spectra in HPLC system	Class
Gallic acid	11.97	254	271		Benzoic acid
Protocatechuic acid	15.38	254	260, 294		Benzoic acid
(-)-Epicatechin	18.55	280	277		Flavan-3-ol
Quercetin-3-*O*-rutinoside	22.02	370	255, 265sh, 350		Flavonol
Ellagic acid	22.30	254	254, 364, 394		Ellagitanin
Luteolin-7-*O*-rutinoside	23.68	370	256, 267sh, 348		Flavone

Vanillic acid	24.55	280	253, 294		Benzoic acid
Kaempferol-3,7-*O*-diglucoside	25.14	370	264, 346		Flavonol
Kaempferol-3-*O*-[6-acetylglucoside]-7-*O*-glucoside	31.63	370	266, 315		Flavonol
Kaempferol-3-*O*-[6-acetylglucoside]-7-*O*-rhamnoside	32.12	370	266, 315		Flavonol

[1] Retention time in HPLC-DAD system of each compound identified in each type of extraction was the same, independently of solvent used in the extraction.
[2]UV bands of individual phenolics found in current study.

6.4.4. Antioxidant activity

6.4.4.1. DPPH scavenging capacity

DPPH scavenging activity of extracts was determined by colorimetry, according to Siddhraju and Becker (2003) (Siddhraju and Becker, 2003), in a 96-microplate wells. 285 µL of freshly DPPH radical solution (4 mg DPPH in 100 mL of 95% of ethanol) was added to each microplate well, followed by 15 µL of each extract. A blank (all reagents and extraction solvent instead of sample) added to one well. Then, microplates were incubated at room temperature, in the dark for 30 min. The absorbance values were recorded at 517 nm wavelengths in a microplate reader (Multiskan™ GO Microplate Spectrophotometer, Thermo Scientific, Vantaa, Finland). The results were expressed as % DPPH radical scavenging activity using the following equation: % DPPH scavenging capacity = (Abs_{blank} - Abs_{sample}/ Abs_{blank}) x 100, where Abs_{blank} means the absorbance value of blank and Abs_{sample} the absorbance value of sample extract.

6.4.4.2. Ferric reducing antioxidant power (FRAP) activity

FRAP activity was determined according to the colorimetric method of Benzie and Strain (1996) (Benzie and Strain, 1996), with small modifications. The assay was conducted in 96 well microplates. A fresh daily FRAP reagent solution was prepared by mixing sodium acetate buffer (300 mM, pH 3.6) with 10 mM TPTZ solution (40 mM HCl as solvent) and 20 mM iron (III) chloride solution in a volume ratio of 10:1:1, respectively. 25 µL of each extract was mixed with 275 µL FRAP solution reagent. Microplates were placed in a dark room temperature for 5 min and then absorbance values were recorded at 593 nm in a microplate reader (MultiskanTM FC Microplate Photometer, USA). $FeSO_4$ (Sigma-Aldrich, Tauferkichen, Germany) was used as control standard and the results were expressed as µmol $FeSO_4$ equivalents g^{-1} dw.

6.4.4.3. Inhibition of lipid peroxidation

Inhibition of lipid peroxidation was determined following the method of Ruberto et al. (2000) (Ruberto et al., 2000), with small modifications, using an egg yolk homogenate as lipid

rich media. To each microplate well 20 µL of egg yolk (10% of egg yolk in 0,1 M phosphate buffer, pH 7.4) was added followed by 5 µL of $FeSO_4$ (1mM), 20 µL of extract and 65 µL of ultrapure water. Microplates were incubated in dark environment for 15 min at 37 ºC. Then, 50 µL of TCA (50%) and 100 µL of TBA (0.8% in phosphate buffer) were added to each microplate well. Microplates were incubated during 15 min at 95ºC and the absorbance values were recorded at 532 nm. For the control, which is a complete oxidized extract (egg yolk + $FeSO_4$, without extract) the same procedure was done. The results were expressed as percentage of inhibition lipid peroxidation, calculated as: Inhibition (%) = [1-(Absorbance sample/Absorbance control)] ×100].

6.5. Statistical analysis

All determinations were performed in triplicate and results were presented as mean ± standard deviation (SD). Data were submitted to statistical analysis by a two-way ANOVA and differences between means were analysed by Tukey test ($p<0.05$). A Pearson´s correlation and a regression analysis were performed in order to evaluate the influence of phytochemicals in the antioxidant activity. All statistical parameter analyses were performed using SPSS Statistics version 24 software (IBM SPSS, Inc., Chicago, USA).

6.6. Results and discussion

6.6.1. Phenolic extraction is cultivar and solvent dependent

A total of 11 phenolics were identified in studied cultivars and grouped in five main classes namely, ellagitannin (ellagic acid), benzoic acids (gallic acid, protocatechuic acid and vanillic acid), flavonols (kaempferol-3,7-*O*-diglucoside, kaempferol-3-*O*-[6-acetylglucoside]-7-*O*-glucoside, kaempferol-3-*O*-[6-acetylglucoside]-7-*O*-rhamnoside and quercetin-3-*O*-rutinoside), flavone (luteolin-7-*O*-rutinoside) and flavan-3-ol (epicatechin). This result is in agreement with the results presented in the past by Shahidi et al. (2007) and more recently by Rusu et al. (2019) for hazelnut kernels, skins, shells and husks, but as expected with different values. Nonetheless, all seems to agree that gallic, ellagic and protocatechuic phenolic acid types are preponderant in this type of plant by-product, as we found in our study. The chemical

structures and typical chromatograms recorded in HPLC-DAD system at different wavelength are presented in Figure 6.1. In Table 6.1., is presented the respective wavelengths of maximum absorption (λ_{max}) (by elution order) of each compound, their retention time and respective UV-DAD spectra, while in Table 6.2. is presented the average content of TPC, TFC and pigments. In Table 6.3. is presented the average amount of each phenolic identified in each extract type. In general, all cultivars shared similar phenolic profile; however, the respective amount of each phenolic varied between cultivar.

According to the results (Table 6.2. and 6.3.), the extraction phenolics yield depends of both cultivar and solvent polarity ($P<0.001$). The highest phenolic contents were achieved in 'Grada de Viseu' cultivar whilst 'Lansing' and 'Butler' showed the lowest levels (Tables 6.3. and 6.4.). In general, the highest amount of phytochemicals (TPC, TFC, TC and individual phenolics), excluding chlorophylls, was obtained under aqueous methanol extraction, followed by water and acetone. Although with hexane it was quantified TPC and TFC, none of individual phenolics found with the previous solvents were detected. This difference could be related with the lowest selectivity exhibited by colorimetry methods and, under 765 and 50 nm wavelength (TPC and TFC content, respectively), other compounds than phenolics are measured interfering with results.

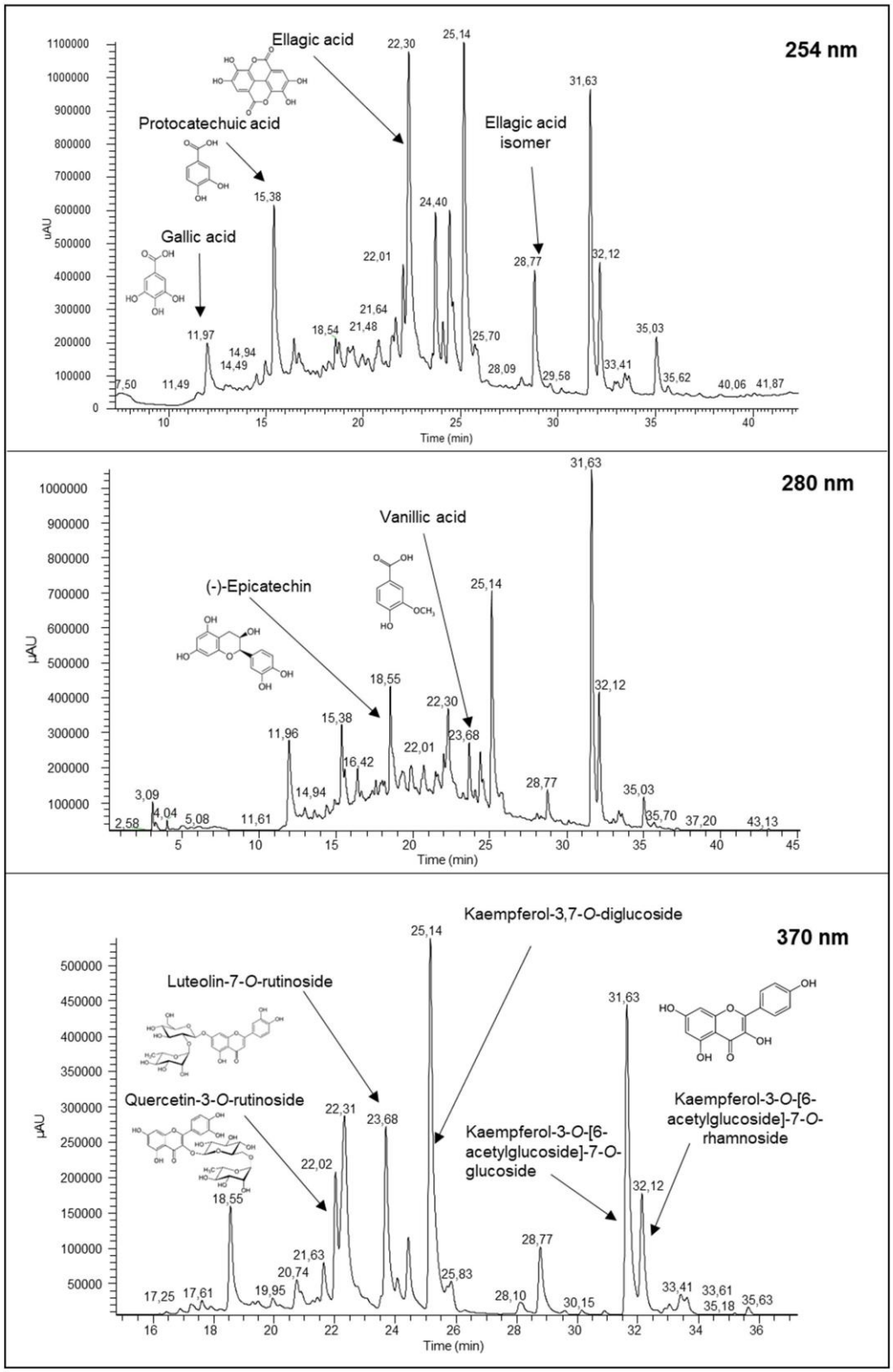


Figure 6.1. Example of typical chromatogram recorded at different wavelength in control samples (‘Grada de Viseu’ cultivar) extracted with aqueous methanol.

Table 6.2. Content of total phenolics (TPC), total flavonoids (TFC), chlorophyll a (Chl a), chlorophyll b (Chl b) and total carotenoids (TC) quantified in husks from four hazelnut cultivars.[1]

Cultivars	Solvent	TPC (mg g^{-1} GAE)	TFC (mg g^{-1} CAE)	Chl a (µg mL^{-1})	Chl b (µg mL^{-1})	TC (µg mL^{-1})
'Butler'	Water	2.20 ± 0.13 d	0.83 ± 0.02 b	0.63 ± 0.03 a	0.72 ± 0.06 a	0.00 ± 0.00 a
	Methanol	10.23 ± 0.14 e	3.82 ± 0.14 d	1.23 ± 0.02 b	1.19 ± 0.01 b	0.62 ± 0.00 b
	Acetone	1.52 ± 0.17 c	1.41 ± 0.12 c	3.15 ± 0.02 d	2.00 ± 0.03 e	0.00 ± 0.00 a
	Ethyl acetate	0.61 ± 0.02 b	0.50 ± 0.01 a	2.94 ± 0.03 d	1.61 ± 0.02 d	0.00 ± 0.00 a
	Hexane	0.22 ± 0.01 a	0.41 ± 0.00 a	1.81 ± 0.28 c	1.47 ± 0.09 c	0.00 ± 0.00 a
	P	***	***	***	***	***
'Grada de Viseu'	Water	2.18 ± 0.31 c	0.67 ± 0.01 a	0.65 ± 0.05 a	0.71 ± 0.06 a	0.00 ± 0.00 a
	Methanol	13.01 ± 1.09 d	3.31 ± 0.27 c	1.63 ± 0.03 b	1.23 ± 0.01 b	0.48 ± 0.00 b
	Acetone	1.64 ± 0.30 bc	1.37 ± 0.25 b	3.43 ± 0.24 c	2.06 ± 0.15 d	0.00 ± 0.00 a
	Ethyl acetate	0.47 ± 0.10 ab	0.45 ± 0.11 a	3.13 ± 0.05 c	1.85 ± 0.07 c	0.00 ± 0.00 a
	Hexane	0.03 ± 0.03 a	0.28 ± 0.08 a	1.55 ± 0.12 b	1.56 ± 0.01 c	0.00 ± 0.00 a
	P	***	***	***	***	***
'Lansing'	Water	2.34 ± 0.04 c	0.87 ± 0.08 b	0.75 ± 0.04 a	0.92 ± 0.03 a	0.00 ± 0.00 a
	Methanol	6.42 ± 0.15 d	2.74 ± 0.06 c	1.20 ± 0.06 b	1.07 ± 0.03 b	0.26 ± 0.00 b
	Acetone	0.52 ± 0.07 b	1.00 ± 0.09 b	2.72 ± 0.03 c	1.74 ± 0.03 c	0.00 ± 0.00 a
	Ethyl acetate	0.46 ± 0.05 b	0.44 ± 0.02 a	2.95 ± 0.01 d	1.89 ± 0.02 d	0.00 ± 0.00 a
	Hexane	0.15 ± 0.12 a	0.33 ± 0.02 a	1.29 ± 0.06 b	1.18 ± 0.11 b	0.00 ± 0.00 a
	P	***	***	***	***	***
'Morell'	Water	0,99 ± 0.10 c	0.29 ± 0.03 a	0.74 ± 0.02 a	0.87 ± 0.04 a	0.00 ± 0.00 a
	Methanol	7.79 ± 0.27 d	2.17 ± 0.04 e	1.55 ± 0.03 b	1.42 ± 0.03 b	0.57 ± 0.02 b
	Acetone	0.59 ± 0.20 bc	0.87 ± 0.08 d	3.29 ± 0.05 d	2.36 ± 0.03 c	0.00 ± 0.00 a
	Ethyl acetate	0.42 ± 0.08 ab	0.62 ± 0.05 c	3.20 ± 0.06 d	2.27 ± 0.05 c	0.00 ± 0.00 a
	Hexane	0.04 ± 0.02 a	0.49 ± 0.02 b	1.82 ± 0.03 c	1.67 ± 0.23 b	0.00 ± 0.00 a
	P	***	***	***	***	***
P	Solvent (S)	***	***	***	***	***
	Cultivar (C)	***	***	***	***	***

[1]Values are expressed as mean ± SE. Results from ANOVA analysis are expressed by the symbol: *** $p < 0.001$. Different letters in each column represents significant differences ($p<0.05$) by Tukey test, between different treatments in each date.

Table 6.3. Content of phenolic compounds quantified in husks from four hazelnut different cultivars (values expressed as mg g-1 dry weight).[1-4]

Cultivars	Solvent	*Benzoic acids*			*Ellagitanins*		*Flavonols*				*Flavone*	*Flavan-3-ols*
		GA	***Proto. A***	***VA***	***EA***	***EA isomer***	***Rut***	***Kaemp-3,7d***	***Kaemp-3-7g***	***Kaemp-3-7r***	***Lut***	***Epic***
‘Butler’	Water	0.25 ± 0.02 b	1.18 ± 0.00 c	0.05 ± 0.00 a	n.d.	n.d.	0.04 ± 0.00 c	0.04 ± 0.00 a	n.d.	n.d.	0.08 ± 0.00 b	0.05 ± 0.00 a
	Methanol	0.36 ± 0.01 c	1.91 ± 0.15 d	0.32 ± 0.02 d	3.06 ± 0.38 b	1.50 ± 0.15 b	0.36 ± 0.00 d	0.77 ± 0.10 b	0.66 ± 0.08 b	0.22 ± 0.03 c	0.79 ± 0.04 d	0.24 ± 0.01 b
	Acetone	0.11 ± 0.01 a	1.08 ± 0.07 b	0.18 ± 0.00 c	0.57 ± 0.13 a	0.50 ± 0.05 a	0.01 ± 0.00 a	0.03 ± 0.01 a	0.02 ± 0.01 a	0.13 ± 0.00 b	0.15 ± 0.02 c	n.d.
	Ethyl acetate	n.d.	0.50 ± 0.02 a	0.07 ± 0.01 b	n.d.	n.d.	0.02 ± 0.00 b	0.03 ± 0.01 a	0.03 ± 0.00 a	0.01 ± 0.00 a	0.03 ± 0.01 a	n.d.
	Hexane	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	P	***	***	***	***	***	***	***	***	***	***	***
‘Grada de Viseu’	Water	0.21 ± 0.04 c	0.82 ± 0.02 b	0.02 ± 0.00 a	1.95 ± 0.00 b	0.52 ± 0.06 b	0.07 ± 0.00 b	0.21 ± 0.01 b	n.d.	n.d.	0.17 ± 0.01 b	0.24 ± 0.00 a
	Methanol	0.61 ± 0.02 d	1.63 ± 0.02 d	0.32 ± 0.01 c	14.39 ± 1.23 d	6.19 ± 0.57 d	0.35 ± 0.02 c	1.66 ± 0.06 d	1.29 ± 0.06 c	0.43 ± 0.02 c	0.90 ± 0.38 d	0.95 ± 0.00 b
	Acetone	0.15 ± 0.01 b	1.07 ± 0.06 c	0.10 ± 0.01 b	4.12 ± 0.59 c	1.62 ± 0.14 c	0.07 ± 0.01 b	0.41 ± 0.03 c	0.66 ± 0.07 b	0.30 ± 0.03 b	0.29 ± 0.04 c	n.d.
	Ethyl acetate	0.03 ± 0.00 a	0.29 ± 0.05 a	n.d.	0.41 ± 0.04 a	0.28 ± 0.00 a	0.01 ± 0.00 a	0.06 ± 0.01 a	0.05 ± 0.00 a	0.03 ± 0.00 a	0.05 ± 0.01 a	n.d.
	Hexane	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	P	***	***	***	***	***	***	***	***	***	***	***

Table 6.3. cont. Content of phenolic compounds quantified in husks from four hazelnut different cultivars (values expressed as mg g-1 dry weight).[1-4]

Cultivars	Solvent	*Benzoic acids*		*Ellagitanins*			*Flavonols*				*Flavone*	*Flavan-3-ols*
		GA	***Proto. A***	***VA***	***EA***	***EA isomer***	***Rut***	***Kaemp-3,7d***	***Kaemp-3-7g***	***Kaemp-3-7r***	***Lut***	***Epic***
‘Lansing’	Water	0.33 ± 0.00 b	1.45 ± 0.01 c	0.07 ± 0.00 b	n.d.	n.d.	n.d.	0.05 ± 0.00 b	n.d.	n.d.	0.06 ± 0.00 c	n.d.
	Methanol	0.63 ± 0.01 c	2.62 ± 0.19 d	0.39 ± 0.05 d	2.88 ± 0.42 b	1.91 ± 0.57 b	0.17 ± 0.02 c	0.60 ± 0.07 d	0.52 ± 0.06 c	0.18 ± 0.02 c	0.30 ± 0.03 d	0.19 ± 0.00
	Acetone	0.09 ± 0.01 a	1.01 ± 0.10 b	0.16 ± 0.01 c	0.27 ± 0.07 a	0.36 ± 0.06 a	0.02 ± 0.00 b	0.09 ± 0.01 c	0.10 ± 0.04 b	0.05 ± 0.00 b	0.04 ± 0.00 b	n.d.
	Ethyl acetate	n.d.	0.42 ± 0.00 a	0.05 ± 0.00 a	n.d.	n.d.	0.01 ± 0.00 a	0.02 ± 0.01 a	0.01 ± 0.01 a	0.01 ± 0.00 a	0.02 ± 0.00 a	n.d.
	Hexane	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	P	***	***	***	***	***	***	***	***	***	***	***
‘Morell’	Water	0.19 ± 0.00 b	0.20 ± 0.01 c	0.02 ± 0.00 a	0.68 ± 0.04 a	0.52 ± 0.04 a	0.02 ± 0.00 a	0.01 ± 0.00 a	n.d.	n.d.	n.d.	0.25 ± 0.00 b
	Methanol	0.37 ± 0.00 c	0.70 ± 0.02 d	0.44 ± 0.02 d	8.02 ± 0.09 b	9.94 ± 1.26 c	0.20 ± 0.02 c	0.61 ± 0.01 c	0.82 ± 0.09 c	0.24 ± 0.00 c	0.68 ± 0.06 c	0.93 ± 0.20 c
	Acetone	0.04 ± 0.00 a	0.17 ± 0.01 b	0.13 ± 0.01 c	0.69 ± 0.06 a	3.08 ± 0.39 b	0.02 ± 0.00 a	0.08 ± 0.01 b	0.20 ± 0.04 b	0.08 ± 0.01 b	0.14 ± 0.08 b	0.02 ± 0.00 a
	Ethyl acetate	n.d.	0.04 ± 0.00 a	0.05 ± 0.01 b	n.d.	0.80 ± 0.18 a	0.03 ± 0.00 b	n.d.	0.02 ± 0.00 a	0.01 ± 0.00 a	0.01 ± 0.00 a	n.d.
	Hexane	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	P	***	***	***	***	***	***	***	***	***	***	***
						ANOVA Analysis						
Cultivar (C)		***	***	***	***	***	***	***	***	***	***	***
Solvent (S)		***	***	***	***	***	***	***	***	***	***	***
C x S		***	***	***	***	***	***	***	***	***	***	***

[1]Abbreviation of phenolic names identified: *GA* (gallic acid), *Proto. A* (protocatechuic acid), *VA* (vanillic acid), *EA* (ellagic acid), *EA isomer* (ellagic acid isomer), *Rut* (quercetin-3-*O*-rutinoside *Kaemp-3,7d* (Kaempferol-3,7-*O*-diglucoside), *Kaemp-3-7g* (Kaempferol-3-*O*-[6-acetylglucoside]-7-*O*-glucoside), *Kaemp-3-7r* (Kaempferol-3-*O*-[6-acetylglucoside]-7-*O*-rhamnoside), *Lut* (luteolin-7-*O*-rutinoside) and *Epic* ((-)-Epicatechin.
[2]Values expressed as mean ± standard deviation (SD) of three replicates.
[3]Probability test values obtained by ANOVA variance analysis; number with different letters in same column are significantly different from another at $p<0.05$ by Tukey test.
[4]The symbols means: highly significant (***) P < 0.001; n.d. (not detected).

Table 6.4. Pearson correlation of all phytochemical parameters with antioxidant capacity in hazelnut husks from four cultivars.[1,2]

Cultivar	Antioxidant activity	TPC	TFC	Chla	Chlb	TC	*GA*	*Proto. A*	*VA*	*EA*	*EA isomer*	*Rut*	*Kaemp-3,7d*	*Kaemp-3-7g*	*Kaemp-3-7r*	*Lut*	*Epic*
Butler	**DPPH**	0.875 *(0.052)*	0.846 *(0.071)*	-0.566 *(0.320)*	-0.501 *(0.390)*	0.769 *(0.129)*	**0.991** ***(0.001)***	**0.974** ***(0.005)***	0.782 *(0.118)*	0.784 *(0.116)*	0.776 *(0.123)*	0.814 *(0.093)*	0.791 *(0.111)*	0.761 *(0.135)*	0.716 *(0.173)*	0.833 *(0.080)*	0.861 *(0.061)*
	FRAP	**0.986** ***(0.002)***	**0.968** ***(0.007)***	-0.473 *(0.421)*	-0.367 *(0.543)*	**0.938** ***(0.018)***	**0.929** ***(0.022)***	**0.927** ***(0.024)***	0.887 *(0.045)*	**0.944** ***(0.016)***	**0.924** ***(0.025)***	**0.958** ***(0.010)***	**0.950** ***(0.013)***	**0.934** ***(0.020)***	0.843 *(0.073)*	**0.969** ***(0.006)***	**0.972** ***(0.006)***
	ILp	0.856 *(0.064)*	**0.944** ***(0.016)***	-0.013 *(0.984)*	0.138 *(0.825)*	0.829 *(0.083)*	0.704 *(0.185)*	0.842 *(0.073)*	**0.990** ***(0.001)***	**0.917** ***(0.028)***	**0.965** ***(0.008)***	0.817 *(0.091)*	0.837 *(0.077)*	0.838 *(0.076)*	**0.997** ***(0.000)***	**0.905** ***(0.034)***	0.782 *(0.118)*
Grada de Viseu	**DPPH**	0.747 *(0.146)*	0.759 *(0.137)*	-0.354 *(0.558)*	-0.547 *(0.340)*	0.632 *(0.253)*	0.863 *(0.059)*	**0.929** ***(0.023)***	0.729 *(0.162)*	0.765 *(0.132)*	0.724 *(0.167)*	0.787 *(0.114)*	0.751 *(0.143)*	0.678 *(0.208)*	0.649 *(0.236)*	0.794 *(0.108)*	0.759 *(0.137)*
	FRAP	**0.999** ***(0.000)***	**0.964** ***(0.008)***	-0.274 *(0.655)*	-0.366 *(0.545)*	**0.978** ***(0.004)***	**0.989** ***(0.001)***	0.857 *(0.063)*	**0.964** ***(0.008)***	**0.982** ***(0.003)***	**0.978** ***(0.004)***	**0.998** ***(0.000)***	**0.987** ***(0.002)***	0.884 *(0.047)*	0.798 *(0.106)*	**0.981** ***(0.003)***	**0.987** ***(0.002)***
	Ilp	0.776 *(0.123)*	**0.909** ***(0.032)***	0.164 *(0.792)*	0.062 *(0.922)*	0.722 *(0.169)*	0.811 *(0.096)*	**0.930** ***(0.022)***	**0.898** ***(0.039)***	0.877 *(0.051)*	0.864 *(0.059)*	0.820 *(0.089)*	0.856 *(0.064)*	**0.956** ***(0.011)***	**0.978** ***(0.004)***	0.886 *(0.045)*	0.679 *(0.207)*
Lansing	**DPPH**	**0.900** ***(0.038)***	0.827 *(0.084)*	-0.574 *(0.311)*	-0.619 *(0.266)*	0.716 *(0.174)*	**0.965** ***(0.008)***	**0.958** ***(0.010)***	0.754 *(0.141)*	0.712 *(0.177)*	0.702 *(0.187)*	0.696 *(0.192)*	0.756 *(0.139)*	0.697 *(0.191)*	0.674 *(0.213)*	0.824 *(0.086)*	0.716 *(0.174)*
	FRAP	**0.967** ***(0.007)***	**0.929** ***(0.023)***	-0.525 *(0.364)*	-0.574 *(0.311)*	0.849 *(0.069)*	**0.995** ***(0.000)***	**0.988** ***(0.002)***	0.870 *(0.055)*	0.849 *(0.069)*	0.841 *(0.074)*	0.836 *(0.078)*	0.881 *(0.048)*	0.838 *(0.077)*	0.817 *(0.091)*	**0.927** ***(0.023)***	0.849 *(0.069)*
	ILp	0.669 *(0.217)*	0.843 *(0.073)*	0.233 *(0.706)*	0.172 *(0.782)*	0.791 *(0.111)*	0.591 *(0.294)*	0.711 *(0.178)*	**0.923** ***(0.026)***	0.836 *(0.078)*	0.876 *(0.052)*	0.855 *(0.065)*	0.841 *(0.074)*	0.881 *(0.049)*	0.917 *(0.028)*	0.792 *(0.110)*	0.791 *(0.111)*
Morell	**DPPH**	**0.906** ***(0.034)***	0.754 *(0.141)*	-0.555 *(0.331)*	-0.576 *(0.309)*	0.860 *(0.062)*	**0.996** ***(0.000)***	**0.957** ***(0.011)***	0.828 *(0.083)*	**0.897** ***(0.039)***	0.835 *(0.078)*	0.872 *(0.054)*	0.862 *(0.061)*	0.832 *(0.081)*	0.807 *(0.099)*	0.840 *(0.075)*	**0.958** ***(0.010)***
	FRAP	**0.997** ***(0.000)***	**0.941** ***(0.017)***	-0.324 *(0.594)*	-0.317 *(0.603)*	**0.985** ***(0.002)***	**0.928** ***(0.023)***	**0.990** ***(0.001)***	**0.968** ***(0.007)***	**0.996** ***(0.000)***	**0.967** ***(0.007)***	**0.989** ***(0.001)***	**0.987** ***(0.002)***	**0.971** ***(0.006)***	**0.951** ***(0.013)***	**0.977** ***(0.004)***	**0.984** ***(0.002)***
	ILp	**0.929** ***(0.023)***	**0.955** ***(0.011)***	0.002 *(0.998)*	0.006 *(0.992)*	**0.937** ***(0.019)***	0.731 *(0.160)*	0.871 *(0.054)*	**0.946** ***(0.015)***	**0.922** ***(0.026)***	**0.931** ***(0.021)***	**0.964** ***(0.008)***	**0.934** ***(0.020)***	**0.931** ***(0.022)***	**0.917** ***(0.028)***	**0.933** ***(0.020)***	0.857 *0.064*

[1]Abbreviation: TPC (total phenolics), TFC (total flavonoids), Chl a (chlorophyll a), Chl b (chlorophyll b), TC (total carotenoids), *GA* (gallic acid), *Proto. A* (protocatechuic acid), *VA* (vanillic acid), *EA* (ellagic acid), *EA isomer* (ellagic acid isomer), *Rut* (quercetin-3-*O*-rutinoside *Kaemp-3,7d* (Kaempferol-3,7-*O*-diglucoside), *Kaemp-3-7g* (Kaempferol-3-*O*-[6-acetylglucoside]-7-*O*-glucoside), *Kaemp-3-7r* (Kaempferol-3-*O*-[6-acetylglucoside]-7-*O*-rhamnoside), *Lut* (luteolin-7-*O*-rutinoside) and *Epic* ((-)-Epicatechin.
[2] In parenthesis is presented the value of p (p <0.05 *, p <0.01 ** and p < 0.001 ***).

Although colorimetry methods are still used by different authors to quantify phytochemicals, we believe that should be used on as an indicator of the phenolic presence in samples. For phenolic quantification, other methods like HPLC-DAD should always be used avoiding any misunderstandings in the results. Considering that the majority of the studies reported in literature (Boulekbache et al., 2013; Fanali et al., 2018) shown a significant influence of solvent in the extraction of phytochemicals, the differences obtained in our study were expected. Phytochemicals and in particularly phenolics, have a wide range of solubility depending upon their structure-guided polarity which was also observed in this study. In fact, under methanol the highest amount of all individual phenolics were observed, followed by water, acetone, ethyl acetate and hexane, which is in agreement with the decreasing polarity of the solvent. Solvents with high polarity extract high content of phenolics (Aires, 2017). Hexane did not extract any phenolics from hazelnut husks, as shown in Table 6.3. These results are in agreement with many studies (Shahidi et al., 2007; Nobossé et al., 2018; Rusu et al., 2019; Elfalleh et al., 2019) in which methanol is the solvent with highest capacity to extract phenolics. Methanol is believed to disrupt the bonding between the solutes and plant matrices which seem to be related to the eluent strength [(water (>1) > methanol (0.95) > acetone (0.6) = ethyl acetate (0.6) > hexane (0.01)] and dielectric constant [water (78.45) > methanol (32.6) > acetone (21.1) > ethyl acetate (6.0) > hexane (1.89)] (Aires, 2017). Although water shown the highest eluent strength (>1) and dielectric constant (78.45) compared to other solvents (Fanali et al., 2018), its ability to remove phenolics from plant matrices seems to be lower when compared with methanol but higher than acetone. It has been widely accepted that high polarity always-mean high extraction and better phenolics solubility into extraction solvent (Elfalleh et al., 2019), however, differences in structures of phenolics can also influence its own solubility. Moreover, factors like extraction temperature, time and method can be critical for phenolic extraction and solubility. Regarding the content of photosynthetic pigments (Table 6.2.), as expected, chlorophylls were better extracted with acetone, whilst carotenoids were exclusively extracted with methanol. Under the other solvents, the average content of carotenoids was vestigial and thus not considered for quantitation. On the other hand, from environmental point of view and considering the toxicity of methanol or acetone, it seems that water might be a valid alternative for extracting phenolics, even if its extraction yield is lower than methanol. The findings from this study showed that aqueous methanol is the best extractant for the phenolics, probably due to the synergism between these two types of solvents.

Regarding the diversity of phenolics, all cultivars showed similar profile but different content (Table 6.3.). In general, the five main phenolics quantified in methanolic extracts of 'Butler' cultivar were ellagic acid and its isomer, protocatechuic acid, Luteolin-7-*O*-rutinoside and kaempferol-3,7-*O*-diglucoside, while in 'Grada de Viseu' were ellagic acid and its isomer, protocatechuic acid, kaempferol-3,7-*O*-diglucoside and kaempferol-3-*O*-[6-acetylglucoside]-7-*O*-glucoside. In methanolic extracts of 'Lansing' ellagic acid and its isomer, protocatechuic acid, gallic acid and kaempferol-3,7-*O*-diglucoside were the five main phenolics quantified, while in 'Morell' cultivar was ellagic acid and its isomer, (-)-epicatechin, kaempferol-3-*O*-[6-acetylglucoside]-7-*O*-glucoside and protocatechuic acid.

This diversity showed that hazelnut husks, often neglected and underused by industry, can be an important source of natural and bioactive compounds with important bioactivities. The ellagic acid found in our samples has been reported by literature as having preventive effect against the occurrence of prostate tumours (Chang et al., 2013) and protective effect against to oxidative damage in hippocampus and brain inflammation (Farbood et al., 2015). Antihyperglycemic, antilipid peroxidative and cardioprotective activity of gallic acid was reported by Punithavathi et al. (2011) (Punithavathi et al., 2011). Anticancer activity through several pathways such as inhibition of angiogenesis and expression of vascular endothelial factors (VEGF), induction of apoptosis, regulation of hypoxia-inducible factor 1-alpha (HIF-1α), induction of G2/M cell cycle arrest, and caspase-3-dependent apoptosis were shown by kaempferol (Huang et al., 2013). Kashafi et al. (2017), found that kaempferol has the ability to increase the apoptosis of human cervical cancer HeLa cells via PI3K/AKT and telomerase pathways. In addition, different studies have shown that a flavonoids rich diet can decrease the risks of breast cancer in women (Labbé et al., 2015) and prostate cancer in men (Yap, 2015). Therefore, not only leaves, kernels, shells and skins from hazelnuts are important sources of bioactive compounds (Esposito et al., 2017; Yuan et al., 2018), but also the hazelnut husks can be important due to its content of important bioactive flavonols as we shown in this study. In addition, 'Grada de Viseu' seems to be the most promising cultivar, although the husks from the other studied cultivars have also important bioactive potential. All these studies shown that phenolics found in the hazelnuts husks turns this material a rich source of polyphenols, that can be used to be in different formulations in cosmetics through reduction of oxidative stress, to treat skin diseases or to accelerate skin wound healing among other inflammations, when applied topically. On the other hand, they can be used as food additives to avoid oxidations of foods or in pharmaceutical industry trough different formulations (supplements, encapsulated)

(Tungmunnithum et al., 2018) for health promoting substances, with antioxidant, antibacterial, anti-cancer, cardioprotective and anti-inflammatory effects, or to boost of immune system, among other effects.

6.6.2. Radical scavenging activity and reducing power of industrial hazelnut by-products phenolics

The results from antioxidant activity assays are shown in Figure 6.2. 'Grada de Viseu' husks presented the highest antioxidant potential by all three methods tested, followed by 'Butler', 'Lansing' and 'Morell'. Methanolic, aqueous and acetone extracts were those with the highest antioxidant activities likewise for phenolics and carotenoids quantification step (Tables 6.2. and 6.3.). This tendency seems to be related with high content of phenolics in these extracts. The results from regression analysis (Figure 6.3.) show a significant and positive correlation between antioxidant activities and TPC, TFC and TC, reinforcing the importance of phenolics and carotenoids in bioactivities of plants extracts and in particularly in the husks of hazelnuts. In a recent study (Fanali et al., 2018), a strong and positive correlation between antiradical activity of hazelnut involucre methanolic extracts and its tannins fraction (gallic + ellagic acids acids) was found, which corroborates our findings. Based in the results it can be stated that methanol is the solvent with the highest capacity to extract phenolics and carotenoids increasing the high antioxidant potential of hazelnut husks. Moreover, results from Pearson´s correlation (Table 6.4.) showed that antioxidant activities in hazelnut husks are more dependent of a combined effect of different phenolics than one or two. Nonetheless, this is highly dependent on the cultivar and method used in the quantification of antioxidant activity. For example, in 'Butler' and 'Lansing' cultivar the phenolics luteolin-7-*O*-rutinoside, gallic and protocatechuic acids showed a stronger influence in antioxidant potential when measured by FRAP method, while in 'Grada de Viseu' were quercetin-3-*O*-rutinoside and gallic acid. In 'Morell', ellagic acid, protocatechuic acid, quercetin-3-*O*-rutinoside and (-)-epicatechin were the most critical (Table 6.4.). The differences observed in the antioxidant activity between the three methods used could be explained by the influence of different compounds in the sample bioactivities. FRAP and Inhibition of lipid peroxidation seems to be more appropriate when phenolics and carotenoids are involved in antioxidant activities, whilst DPPH seems to be more appropriate when other compounds than phenolics are preponderant.

Although extraction with water lead to lower phenolic and carotenoid extraction yields and antioxidant activities compared to methanol, it could be a reliable alternative to extract bioactive compounds from hazelnut husks due to its nontoxic effect in the environment and safeness for consumers.

Therefore, based in the herein presented results hazelnut husks, often neglected by producers, are a reliable source of phytochemicals and thus they could be used to isolate natural compounds for cosmetic, pharmaceutical or food industries.

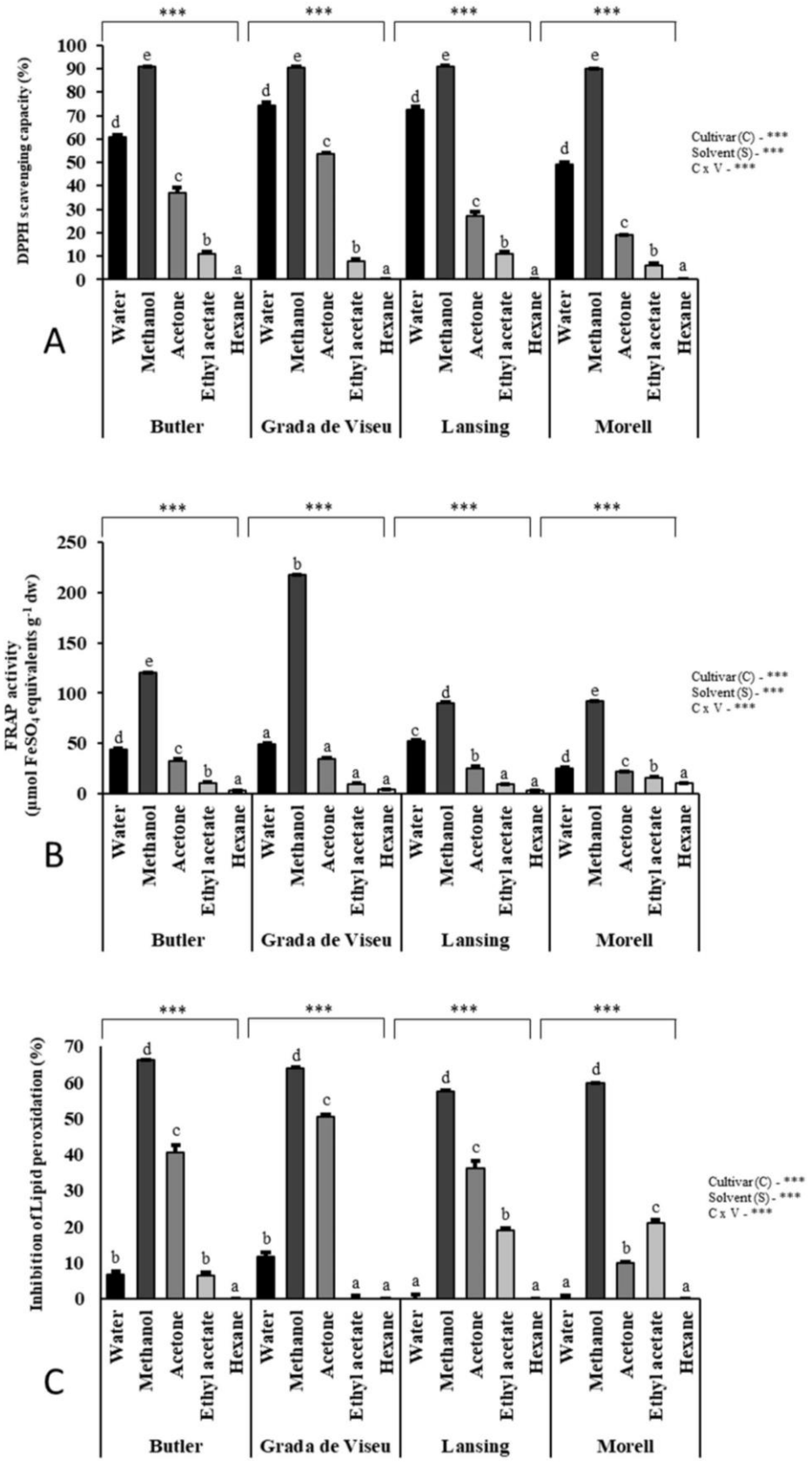


Figure 6.2. Antioxidant activity results of hazelnut husks obtained by three different quantification methods.

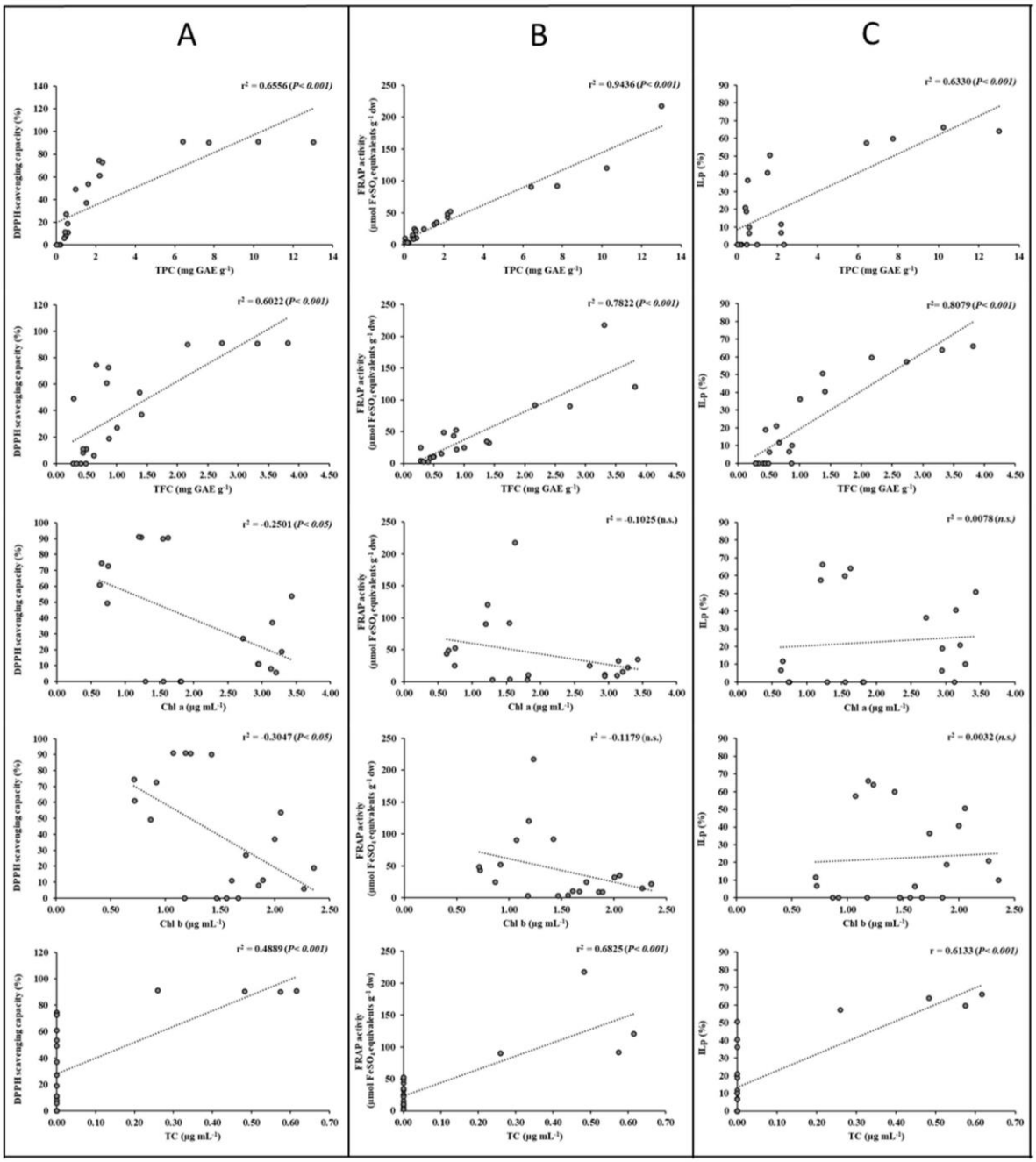


Figure 6.3. Regression of total phenolics (TPC), total flavonoids (TFC), chlorophyll a (Chl a), chlorophyll b (Chl b) and total carotenoids (TC) with DPPH (A), FRAP (B) and inhibition of lipid peroxidation (ILP) (C) activities. Statistical differences were according to the analysis of variance and Tukey's test. Statistical differences were set at $p < 0.05$, $p < 0.01$ and $p < 0.001$.

6.7. Conclusion

Cultivar and extraction solvent, as well as the interaction between them, influence the phytochemical content and antioxidant activity of hazelnut husks. Eleven phenolics were identified with variable content and ellagic acid was the phenolic with the highest content in all cultivars. Methanol was the most efficient solvent for the production of polyphenol-rich extracts compared to other solvents. However, water can be considered a safe and environmental friendly alternative to methanol. From all cultivars, hazelnut husks from 'Grada de Viseu' seems to be the most promising to produce extracts with higher bioactive value.

Acknowledgements

The author Sandra Cabo acknowledges the financial support by the Portuguese Foundation for Science and Technology (FCT) (PB/BD/113615/2015) under the Doctoral Programme "Agricultural Production Chains – from fork to farm" (PD/00122/2012). The authors also acknowledge the financial support provided by National Funds from FCT, under the project UID/AGR/04033/2019. The authors acknowledge the financial support of INTERACT project "Integrative Research in Environment, Agro-Chains and Technology", no. NORTE-01-0145-FEDER-000017, in its line of research entitled ISAC, co-financed by the European Regional Development Fund (ERDF) through NORTE 2020 (North Regional Operational Program 2014/2020) and Project IBERPHENOL, Project Number 0377_IBERPHENOL-6-E, co-financed by European Regional Development Fund (ERDF) through POCTEP 2014-2020.

Conflict of interest

The authors declare no conflict of interest.

6.8. References

Aires, A., Carvalho, R., Rosa, E.A.S. and Saavedra, M.J. 2013.Phytochemical characterization and antioxidant properties of organic baby-leaf watercress produced under organic production system. *CyTA-J Food,* 11, 343-351.

Aires, A. 2017. Phenolics in foods: extraction, analysis and measurements. In: Phenolic Compounds - Natural Sources, Importance and Applications, Marcos Soto-Hernandez, Mariana Palma-Tenango and Maria del Rosario Garcia-Mateos (Eds.), ISBN 978-953-51-2958-5, IntechOpen, London, SE19SG-United Kingdom. pp. 61-88.

Bacchetta, L., Rovira, M., Tronci ,C., Aramini, M., Drogoudi, P., Silva, AP., Solar, A., Avanzato, D., Botta, R., Valentini, N., and Boccacci, P. 2005. A multidisciplinary approach to enhance the conservation and use of hazelnut *Corylus avellana* L. genetic resources. *Genet. Resour. Crop. Evol*., 62, 649–663.

Benzie, I.F.F., and Strain, J.J. 1996. The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": The FRAP assay. *Anal. Biochem.,* 239, 70–76.

Bouayed, J., and Bohn, T. 2010. Exogenous antioxidants—double-edged swords in cellular redox state: health beneficial effects at physiologic doses versus deleterious effects at high doses. *Oxid. Med. Cell. Longev*., 3, 228–237.

Boulekbache-Makhlouf, L., Medouni, L., Medouni-Adrar, S., Arkoub, L., and Madani, K. 2013. Effect of solvents extraction on phenolic content and antioxidant activity of the byproduct of eggplant. *Ind. Crops. Prod.,* 49, 668-674.

Chang, Y., Chou, D-S., Sheu, J-R., Chen, W-F., Lin, K-H., Hsieh, C-Y., Lin, L-J., and Chang, C-C. 2013. Novel bioactivity of ellagic acid in inhibiting human platelet activation. *Evid. Based. Complement. Alte.r Med.,* 1–9.

Çöpür, Y., Güler, C., Akgül, M., and Taşçioğlu, C. 2007. Some chemical properties of hazelnut husk and its suitability for particleboard production. *Build. Environ*., 42, 2568–2572.

Dewanto, V., Wu, X., Adom, K.K., and Liu RH. 2002. Thermal processing enhances the nutritional value of tomatoes by increasing total antioxidant activity. *J. Agr. Food. Chem*. 50, 3010-3014.

Elfalleh, W., Kirkan, B., and Sarikurkcu, C. 2019. Antioxidant potential and phenolic composition of extracts from *Stachys tmolea*: An endemic plant from Turkey. *Ind. Crops. Prod*., 27, 212–216.

Esposito, T., Sansone, F., Franceschelli, S., Del Gaudio, P., Picerno, P., Aquino, RP., and Mencherini, T. 2017. Hazelnut (*Corylus avellana* L.) shells extract: Phenolic composition, antioxidant effect and cytotoxic activity on human cancer cell lines. *Int. J. Mol. Sci*., 18.

Fanali, C., Tripodo, G., Russo M., Della Posta S., Pasqualetti V., and De Gara L. 2018. Effect of solvent on the extraction of phenolic compounds and antioxidant capacity of hazelnut kernel. *Electrophoresis*., 39(13), 1683-1691.

FAO. 2016. Agricultural Production Crops – Hazelnut. Food and Agriculture Organization of the United Nations. Access online: http://faostat.fao.org.

Farbood, Y., Sarkaki, A., Dianat, M., Khodadadi, A., Haddad, M.K., and Mashhadizadeh S. 2015. Ellagic acid prevents cognitive and hippocampal long-term potentiation deficits and brain inflammation in rat with traumatic brain injury. *Life Sci*., 124, 120–127 (2015).

Guney, MS. 2013. Utilization of hazelnut husk as biomass. *Sustain. Energy. Technol. Assessments*., 4,72–77.

Huang, W.W., Tsai, S.C., Peng, S.F., Lin, M.W., Chiang, J.H., Chiu, Y.J., Fushiya, S., Tseng, MT., and Yang, J.S. 2013. Kaempferol induces autophagy through AMPK and AKT signalling molecules and causes G 2 /M arrest via downregulation of CDK1/cyclin B in SK-HEP-1 human hepatic cancer cells. *Int. J. Oncol.*, 42,2069–2077.

Ignat I., Volf I., and Popa, V.I. 2011. A critical review of methods for characterisation of polyphenolic compounds in fruits and vegetables. *Food Chem.*, 26, 1821–1835.

John, K.M.M., Harnly, J., and Luthri D. 2018. Influence of direct and sequential extraction methodology on metabolic profiling. *J. Chromat. B.*, 1073, 34-42.

Kashafi, E., Moradzadeh, M., Mohamadkhani, A., and Erfanian S. 2017. Kaempferol increases apoptosis in human cervical cancer HeLa cells via PI3K/AKT and telomerase pathways. *Biomed. Pharmacother*., 89, 573–577.

Kottek, M., Grieser, J., Beck, C., Rudolf, B., and Rubel, F. 2006. World Map of the Köppen-Geiger climate classification updated. *Meteorologische Zeitschrift*, 15(3), 259–263

Labbé, D.P., Zadra, G., Ebot, E.M., Mucci, L.A., Kantoff, P.W., Loda, M., and Brown, M. 2015. Role of diet in prostate cancer: The epigenetic link. *Oncogene*., 34, 4683–4691.

Li, A.N., Li, S., Zhang, Y.J., Xu, X.R., Chen, Y.M., and Li, H.B. 2014. Resources and biological activities of natural polyphenols. *Nutrients.*, 6, 6020–6047.

Li, F., Li, S., Li, H.B., Deng, G.F., Ling, W.H., Wu, S., Xu, X.R., and Chen, F. 2013 Antiproliferative activity of peels, pulps and seeds of 61 fruits. *J. Funct. Foods*., 5,1298–1309.

Lichtenthaler, H.K., and Wellburn, A.R. 1983. Determination of total carotenoids and chlorophylls a and b of leaf in different solvents. *Bio.l Soc. Trans*., 11, 591-592.

Lopez-Calleja, I.M., Cruz, S.D., La Pegels, N., Gonzalez, I., Garcia, T., and Martin, R. 2005. High resolution TaqMan real-time PCR approach to detect hazelnut DNA encoding for ITS rDNA in foods. *Food Chem.,* 141, 1872–1880.

Masullo, M., Cerulli, A., Mari, A., de Souza Santos, CC., Pizza, C., and Piacente, S. 2017. LC-MS profiling highlights hazelnut (Nocciola di Giffoni PGI) shells as a byproduct rich in antioxidant phenolics. *Food Res. Int.,* 101, 180–187.

Mojzer, B.E., Knez, H.M., Škerget, M., Knez, Ž., and Bren, U. 2016. Polyphenols: extraction methods, antioxidative action, bioavailability and anticarcinogenic effects. *Molecules.,* 21(7), 901.

Molnar, T.J., Goffreda, J.C., and Funk, C.R. 2005. Developing hazelnuts for the eastern United States. *Acta Hortic*., 686, 609–618.

Nobossé, P., Fombang, E.N., and Mbofung, C.M.F. 2018. Effects of age and extraction solvent on phytochemical content and antioxidant activity of fresh *Moringa oleifera* L. leaves. *Food Sci. Nutr.,* 6, 2188–2198.

Punithavathi, V.R., Prince, P.S.M., Kumar, R., and Selvakumari, J. 2011. Antihyperglycaemic antilipid peroxidative and antioxidant effects of gallic acid on streptozotocin induced diabetic Wistar rats. *Eur. J. Pharmacol*., 650,465–471.

Ruberto, G., Baratta, M.T., Deans, S.G., and Dorman, H.J.D. 2000. Antioxidant and antimicrobial activity of *Foeniculum vulgare* and *Crithmum maritimum* essential oils. *Planta Med*., 66, 687–693.

Rusu, M.E., Fizesan, I., Opo, A., Gheldiu, A-M., Mocan, A., Crisan, G., Vlase, L., Loghin, F., Popa, D-S., and Tomuta, I. 2019. Enhanced recovery of antioxidant compounds from hazelnut (*Corylus avellana* L.) involucre based on extraction optimization: phytochemical profile and biological activities. *Antioxidants.,* 8, 460.

Shahidi, F., Alasalvar, C., and Liyana-Pathirana, C.M. 2007. Antioxidant phytochemicals in hazelnut kernel (*Corylus avellana* L.) and hazelnut byproducts. *J. Agr. Food Chem.,* 55, 1212–1220.

Siddhraju, P., and Becker, K. 2003. Antioxidant properties of various solvents extracts of total phenolic constituents from three different agroclimatic origins of drumstick tree (*Moringa oleifera* Lam) leaves. *J. Agr. Food Chem.,* 51,2144–2155.

Singleton, V.L., and Rossi, JA. 1965. Colorometry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. *Am. J. Enol. Vitic.*, 16,144-158.

Tungmunnithum, D., Thongboonyou, A., Pholboon, A., and Yangsabai, A. 2018. Flavonoids and other phenolic compounds from medicinal plants for pharmaceutical and medical aspects: An Overview. *Medicines* (Basel, Switzerland). 5, 93.

Uzuner S., and Cekmecelioglu, D.2014. Hydrolysis of hazelnut shells as a carbon source for bioprocessing applications and fermentation. *Int. J. Food. Eng*., 10,799–808.

Yap, S. 2015. Reversing breast cancer in a premenopausal woman: a case for phyto-nutritional therapy. *Int. J. Biotechnol*., Wellness Ind. 4,25–39.

Yuan, B., Lu M., Eskridge, K.M., Isom, L.D., Hanna, M.A. 2018. Extraction, identification, and quantification of antioxidant phenolics from hazelnut (*Corylus avellana* L.) shells. *Food Chem*., 244, 7–15.

6.9. Additional files

Supplementary Table. Monthly average daily air temperature and accumulated precipitation in 2016, in Vila Real region, North of Portugal.

	Months											
	January	February	March	April	May	June	July	August	September	October	November	December
Temperature (ºC)	7.87	6.91	7.72	10.36	13.55	18.40	23.11	22.66	18.90	13.78	8.37	5.58
Precipitation (mm)	115.20	283.60	91.80	193.00	124.40	25.20	0.20	0.20	28.40	81.80	151.80	44.60