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Extraction and Characterization of Biomolecules from Agricultural Wastes

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Chestnut (*Castanea sativa* Mill.) and hazelnut (*Corylus avellana* L.) shells product from farming and food industry processes, because of their valuable biomolecules content, were analysed **as** promising source of antioxidant compounds. The aim of this study was to evaluate several extraction procedures of such agricultural by-products and to characterize specific phenolic molecules contained in the shells of chestnuts and hazelnuts. For these purpose we used different extraction solvents, such as water, methanol/H_2O 70/30(v/v), and absolute methanol.
Specifically, the extraction yield, the content of total polyphenols by Folin-Ciocalteu colorimetric assay, and the antioxidant activity by FRAP (Ferric Reducing Antioxidant Power) were measured. In particular the following specific phenolic compounds were detected and determined by HPLC analysis: gallic, chlorogenic, ferulic, *p*-coumaric, and ellagic acids, (+)-catechin, (–)-epicatechin, and rutin. The values of extraction yield and total polyphenol content decreased according to the extraction solvent in the following order: absolute methanol > 70% methanol > water. The antioxidant activity showed the opposite trend among the three types of extraction. The values obtained from the analysis were higher in chestnut rather than in hazelnut shell.
The specific amount of phenolic compounds varied depending on the solvent used for the extraction. Gallic acid was found in all samples extracted, though with lower values in hazelnut shells treated in water. High values of (–)-epicatechin, chlorogenic acid and ellagic acid were found in hazelnut samples extracted with hydro-alcoholic solution and water. A fair amount of catechin was detected in all samples, whilst rutin, *p*-coumaric acid and ferulic acid were present in small amounts, although the best results were obtained in absolute methanol. The results of this study demonstrated that with a simple extraction process considerable amount of phenolic compounds with high antioxidant activity were obtained from chestnut and hazelnut shells.

1. Introduction

Chestnut (*Castanea sativa* Mill.) and hazelnut (*Corylus avellana* L.) are two widely diffused crops in European and Asiatic countries. The kernel of hazelnut and chestnut are typically consumed whole (raw, boiled, or roasted, without skin) or used as ingredient in a variety of processed foods, especially in bakery and confectionery products. The agricultural and industrial processing of these plant foods results in the production of by-products that are rich sources of bioactive compounds, including polyphenolic compounds with high antioxidant activity (Moure et al., 2001).

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Several studies have investigated the presence of bioactive antioxidant molecules, not only in edible kernels of chestnut (Barreira et al., 2008; Nazzaro et al., 2011; Vekiari et al., 2008) and hazelnut (Delgado et al., 2010; Shahidi et al., 2007), but also in chestnut waste (De Vasconcelos et al., 2010; Vázquez et al., 2008) and hazelnut waste (Contini et al., 2008; Del Rio et al., 2011). There is strong evidence that polyphenols provide protection against harmful effects of free radicals and are known to reduce the risk of several diseases including certain types of cancer, coronary heart disease (CHD), type-2 diabetes, and inflammation. The action of polyphenols on health is also to protect against environmental stresses (Yurtass et al., 2000).

Nowadays, the need to replace synthetic antioxidants used in the pharmaceutical, cosmetic and food industries (i.e. BHT, BHA, TBHQ), whose safety has been questioned, has promoted the research on new sources of antioxidant molecules (Moure et al., 2001), so undervalued agricultural or industrial by-products could be an inexpensive supply of these compounds. Extracts of natural antioxidants from hazelnut skin could potentially be used as nutraceuticals and dietary supplements.

In addition, the hazelnut and chestnut shells absorption of heavy metals present in the industrial waste water was proposed (Liu et al., 2008). Previous studies related the use of hazelnut and chestnut shell as promising biosorbents for copper removal from aqueous solutions (Pehlivan et al., 2009; Yao et al., 2010). Hazelnut shell is mostly used as fuel for burning, mulching and as raw material for production for furfural in the dye industry (Stévigny et al., 2007). Extracts from chestnut tissues could be used as phenol substitutes in the formulation of adhesives, as chrome substitutes in leather tanning and even as antioxidant supplements in food (Vazquez et al., 2008, 2009).

The aim of this study was to evaluate different extraction procedures of such agricultural by-products and to characterize specific polyphenolic molecules contained in the shells of chestnuts and hazelnuts.

2. Experimental

2.1 Sampling and pretreatment

Chestnut (*C. sativa* Mill.) shells were obtained from a local farm at Montella (Avellino, Italy). Hazelnuts (*C. avellana* L.) were harvested in Calvi (Benevento, Italy). For hazelnut samples, the shells were manually separated from the kernel, and ground in a coffee grinder (mod. CBG5 series, Black and Decker Canada Inc., Brockville, ON). Powdered samples were defatted using *n*-hexane (ratio of solid/liquid 1/10, w/w) at room temperature, and the solvent was evaporated in a rotavapor (HeiVap-Heidolph, Germany).

2.2 Extraction and concentration

The extraction of phenolic compounds was carried out using solvents at different polarity: absolute methanol, methanol-water 70/30 (v/v) solution, and water. Powdered defatted sample and solvent (ratio of solid/liquid 1/10, w/w) were mixed and kept at room temperature, for 5 days, under constant stirring. The mixture was centrifuged at 4000 rpm for 15 min, and the supernatant was filtered through a filter paper. Than, the solvent was evaporated in a rotavapor. The extraction yield was expressed as dry matter percentage.

2.3 Total phenols content

Total polyphenols analysis was performed by the colorimetric method, as described by Vázquez et al. (2008), with some modifications. The sample was redissolved in the extraction medium. To 100 µL of sample, 500 µL of Folin-Ciocalteu reactive, and 400 µL of 7.5 % aqueous solution of Na_2CO_3 were added. The mixture was kept for 90 min in the dark at room temperature. The absorbance was read at 720 nm using a UV/Vis spectrophotometer (Model DU 730, Beckman Coulter, Brea, CA). Gallic acid (5–40 mM) was used for constructing the standard curve, and the results were expressed as g of gallic acid equivalents (GAE)/100 g of extract.

2.4 Antioxidant activity

The antioxidant activity of samples was determined by the FRAP assay, according to Szöllösi and Szöllösi-Varga (2002). Dry extract was resuspended in 200 µL of ultrapure water. To 50 µL of sample, 1.5 mL of freshly prepared FRAP reagent (50 mL of 300 mM acetate buffer, pH 3.6; 5 mL of 10 mmol

TPTZ in 40 mM HCl; 5 mL of 20 mM $FeCl_3 \cdot 6H_2O$) were added. The absorbance was recorded at 593 nm after 5 min. The antioxidant activity was calculated from the calibration curve of ascorbic acid (0.1–1 mM). Results were expressed as nmol of ascorbic acid equivalents (AAE)/mg of extract.

2.5 HPLC analysis

Phenolic compounds analysis was performed by HPLC/UV with a C18 Hypersil (Thermo Electron Corporation, Bellefonte, PA) column. The injected sample volume was 20 µL. The solvent flow rate was 0,9 mL/min and the mobile phase was a four-step linear solvent gradient system (0–5 min, 10 % B; 5–40 min, 45 % B; 40–45 min, 100 % B; 45–50 min, 100 % B; 50–55 min, 10 % B) using 2 % acetic acid in water as solvent A, and 0.5% acetic acid in 50% acetonitrile as solvent B. UV detection was carried out at 280 nm. The pure standards utilized were: gallic, ferulic, ellagic, chlorogenic, and *p*-coumaric acid, (+)-catechin, (–)-epicatechin and rutin,. Results were expressed as µg polyphenol compound/mg of extract.

3. Results and discussion

3.1 Extraction yield

The results of the extraction yields are presented in Table 1. For all used solvents, the chestnut shells provided higher extraction yields than the hazelnut shell. The largest yield value for chestnut shell was 9.29 %, obtained with absolute methanol, while the lowest result was 2.20 %, obtained using water as solvent. The yields of hazelnut shell were 2.30 %, 0,65 %, and 0.29 %, corresponding to extraction with 70 % methanol, absolute methanol, and water, respectively.

These results disagree with literature data, which reported that the yield of extractable compounds increased with the polarity of the solvent, so the extraction with water had the highest yield (Vázquez et al., 2008). Instead the extraction from hazelnut shell with 70 % methanol produced similar results to those obtained by Contini et al.(2008).

3.2 Total phenols content and antioxidant activity of the extracts

The total phenols content (Table 1) was widely higher for chestnut shell extracts, when compared to hazelnut shell extracts, for all types of solvent of extraction. The values for chestnut shell were 33.32, 42.08, and 49.14 g GAE/100 g extract, for extraction with water, 70 % methanol, and absolute methanol, respectively. The content of total phenols in hazelnut wastes ranged from 9.95 g GAE/100 g extract with 70% methanol to 14.23 g GAE/100 g extract with absolute methanol. The higher content of total polyphenols was obtained using methanol as solvent, for both chestnuts and hazelnut waste samples. The obtained results are in contrast with those reported by Vázquez et al.(2008), which measured larger amounts of total phenols extracted with water (55.8 g GAE/100 g extract) than those obtained with methanol (32.5 g GAE/100 g extract) from chestnut shell.

The disagreement with literature data could be due to the differences in the studied cultivar, stadium of fruits' harvesting and local climatic conditions.

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Barreira et al. (2008) measured the total phenols content of various parts of chestnut plant (flowers, leaves, outer skin, inner skin, and fruit). In spite of the low values of yield extraction, the phenols

Table 1: Extraction yield, total polyphenols, and antioxidant activity of chestnut and hazelnut shells.

Sample	Extraction yield (%)	Total polyphenols (g GAE/100 g extract)	Antioxidant activity (nmol AAE/mg extract)
Chestnut shell			
CH_3OH	9.29 ±0.15	49.14 ±0.82	2268 ±5.17
CH_3OH/ H_2O 70:30	4.02 ±0.38	42.08 ±2.47	3779 ±180.23
H_2O	2.20 ±0.31	33.32 ±4.65	3035 ±78.82
Hazelnut shell			
CH_3OH	0.65 ±0.02	14.23 ±0.47	545 ±60.58

CH_3OH/ H_2O 70:30	2.30 ±0.25	9.95 ±1.09	415 ±34.96
H_2O	0.29 ±0.10	12.75 ±0.38	351 ±1.72

content was high, indicating the efficiency of the extraction performed using water at boiling temperature for 30 min.

In fact, a significant content of total phenols (> 450 mg/g extract) was found in the peel and in the outer skin of sweet chestnut, and this seems due to an abundance of tannins (Hwang et al., 2001).

The disagreement to Barreira's data could be attributed to the difference in the raw materials; in the present work, mixtures of chestnut shell and inner skin obtained from manufacturing processes were supplied from farm.

The phenolic content of hazelnut shell (9.95 g/100g) resulted greater than the value (5.66 g/100g) reported by Contini et al. (2008), which performed the extraction by long maceration at room temperature, using 80 % methanol as solvent. The amount and quality of phenolic molecules by extraction in solvents depends on the plant material, the solvent used (Marinova et al. 1997; Moure et al., 2000), as well as the contact time of extraction (Delgado et al., 2010).

Regarding the antioxidant activity (Table 1), there are substantial differences among chestnut and hazelnut extracts. The values for FRAP assay varied from 2268 to 3779 nmol AAE/ mg extract for chestnut shell, and from 351 to 545 nmol AAE/ mg extract for hazelnut shell. Results revealed that the highest antioxidant activity for chestnut shell was obtained with 70% methanol, while the lowest value was obtained with absolute methanol. Moreover, the highest antioxidant activity for hazelnut wastes were measured in samples treated with absolute methanol, and the lowest one corresponded to the samples treated with water.

In a previous study (Vázquez et al., 2008), the antioxidant activity of extract from chestnut shell treated with methanol (475 nmol AAE /mg extract), were much lower than our values (2268 nmol AAE /mg extract), while data for extraction with water (3555 nmol AAE /mg extract) were similar to our results. Vázquez et al. obtained the highest antioxidant activity for chestnut shell by extraction with 50 % methanol (3808 nmol AAE /mg extract); while a more large increase in antioxidant activity was observed in extracts treated with alcohol-water solutions, when compared to those treated with pure solvents.

3.3 HPLC analysis

Several phenolic compounds (Table 2) were detected by HPLC/UV analysis. As for gallic acid, the highest value (9.31 µg/mg extract) was measured for chestnut shell, using water as solvent, while the lowest value (0.16 µg/mg extract) was observed for hazelnut shell extracted with the same type of solvent. Little difference was found between the two types of wastes, when 70 % methanol was used.

The concentration of gallic acid in hazelnut shell treated with 70 % methanol was higher than extraction with 80% ethanol (Shahidi et al., 2007), as the efficiency of extraction by ethanol and methanol was similar for hazelnut shell treatment (Contini et al., 2008). The concentration of gallic acid in the chestnut waste, referred to the defatted samples, and treated with 70 % methanol, are in accordance with the range of values (0.14 to 0.33 mg/g) reported by De Vasconcelos et al. (2010).

Table 2: Specific polyphenols in extracts of chestnut and hazelnut shells (µg/mg extract).

	GA	**ChA**	***p*-CA**	**EA**	**FA**	**C**	**EC**	**R**
Chestnut shell								
H_2O	9.31	0.33	0.16	0.54	0.01	0.68	0.07	nd
CH_3OH/ H_2O 70:30	4.53	0.54	0.23	1.05	0.20	1.33	0.95	0.05
CH_3OH	5.84	0.02	nd	0.04	nd	0.38	0.11	nd
Hazelnut shell								
H_2O	0.16	0.76	0.15	0.46	0.16	1.11	0.16	0.13
CH_3OH/ H_2O 70:30	3.52	2.23	0.51	3.98	0.72	0.51	6.62	0.24
CH_3OH	2.71	2.63	0.16	3.29	0.41	1.05	5.52	0.04

GA = gallic acid; ChA = chlorogenic acid; *p*-CA = *p*-coumaric acid; EA = ellagic acid; FA = ferulic acid; C = (+)-catechin; EC = (–)-epicatechin; R = rutin; nd = not detected.

The highest content of ellagic acid was found in hazelnut shell extracted with 70% methanol (3.98 µg/mg extract), and with absolute methanol (3.29 µg/mg extract). All other samples contained ellagic acid in the range 0.04–1.05 µg/mg extract. It is remarkable that chestnut shell has lower content ellagic acid, when compared to hazelnut shell. The amount of ellagic acid obtained by 70 % methanol is outside the range of values (0.14–0.18 mg GAE/g) previously reported (De Vasconcelos et al., 2010).
A consistent presence of chlorogenic acid was found in the extracts of hazelnut shell, using methanol as solvent, both absolute (2.63 µg/mg extract) and 70 % solution (2.23 µg/mg extract). Other results ranging from 0.02 µg/mg extract (chestnut shell in methanol) to 0.76 µg/mg extract (hazelnut shell in water).
The amount of *p*-coumaric acid was scant in almost all types of extracts, and it was not detected in the extract of chestnut waste treated with pure methanol. The highest value was found in the hazelnut waste extracted with 70 % methanol, with 0.51 µg/mg extract, a value that is slightly lower than those referred by Shahidi et al. (2007).
The highest amount of ferulic acid was 0.81 µg/mg extract, corresponding to chestnut shell extracted with water, while this compound was not detected in chestnut waste obtained after absolute methanol treatment. Ferulic acid was present in all the samples of hazelnut shell, with the largest value of 0.72 µg/mg extract, obtained through 70 % methanol extraction. This result exceeds twice those reported in literature (Shahidi et al., 2007).
As regard chestnut shell, rutin was not detected in samples extracted with water and absolute methanol, while an amount of 0.05 µg/mg extract were measured with 70 % methanol treatment. In hazelnut shell, the range of concentration was 0.04-0.24 µg/mg extract, and the efficiency of solvent increased in the following order: absolute methanol > water > 70 % methanol.
The lowest concentration of (+)-catechin in hazelnut shell was measured in sample treated with 70% methanol (0.51 µg/mg extract), while the other two treatments led to similar results. Instead, the extraction with 70% methanol was more effective on chestnut shell (1.33 µg/mg extract), when compared to the treatment in absolute methanol or water.
A high content of (–)-epicatechin was obtained from hazelnut shell treated in absolute methanol (5.52 µg/mg extract) and in hydro-alcoholic solution (6.62 µg/mg extract). The amount of (–)-epicatechin in chestnut shell ranged between 0.07 and 0.95 µg/mg extract, values corresponding to the samples extracted in water and in 70 % methanol, respectively.
Owing to the chemical characteristics of the solvent and also to the diverse structural compositions of the natural products, there is not a single method or extraction solvent system for fruit or vegetable phenolic material. In this study we observed that a solvent can be selective for certain types of molecules, but not for others, and each material-solvent system may show a different behavior in the extraction of polyphenols from agro-industrial wastes.

4. Conclusions

Chestnut and hazelnut waste extracts were obtained using solvents at different polarity. The results of this study demonstrated that considerable amount of polyphenolic compounds with high antioxidant activity were obtained from chestnut and hazelnut shells when a suitable extraction process was performed.
According to the results of this work, we can conclude that the chestnuts waste phenolic extracts show as extraction yield as antioxidant activity higher than hazelnut waste phenolic extracts, so they could be more interesting for food, cosmetics and / or pharmaceutical industry.

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