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S3.03. ANTIOXIDANT ACTIVITY OF CHESTNUT CONSTITUENTS: FLOWERS, LEAVES, SKINS AND FRUITS

[1]João C.M. Barreira, [1,*]Isabel C.F.R. Ferreira, [2]M. Beatriz P.P. Oliveira, [1]José Alberto Pereira

[1] CIMO/Escola Superior Agrária, Instituto Politécnico de Bragança, Campus de Santa Apolónia, Apartado 1172, 5301-855 Bragança, Portugal.
[2] REQUIMTE/Serviço de Bromatologia, Faculdade de Farmácia da Universidade do Porto, Rua Aníbal Cunha, 164, 4099-030 Porto, Portugal.
*Tel +351-273 303219; Fax +351-273 325 405; e-mail: iferreira@ipb.pt

ABSTRACT

In this study, the antioxidant properties of different chestnut constituents (flowers, leaves, skins and fruits) were evaluated through several biochemical assays: DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging activity, reducing power, inhibition of β-carotene bleaching, inhibition of oxidative hemolysis in erythrocytes, induced by 2,2'-azobis(2-amidinopropane)dihydrochloride (AAPH), and inhibition of lipid peroxidation in pig brain tissue through formation of thiobarbituric acid reactive substances (TBARS). These assays have been extensively studied as models for the peroxidative damage in biomembranes. For all the methods EC_{50} values were calculated in order to evaluate the antioxidant efficiency of each product. The phenol and flavonoid contents were also obtained and correlated to antioxidant activity. Chestnut skins revealed much better antioxidant properties, presenting much lower EC_{50} values, particularly for lipid peroxidation inhibition in TBARS assay. Also, the highest antioxidant contents (phenols and flavonoids) were found for this constituent.

Key-words: Chestnut constituents; Antioxidants; Scavenging effects; Peroxidation and Hemolysis inhibition

1. INTRODUCTION

Free radicals were a major interest for early physicists and radiologists and much later found to be a product of normal metabolism. Today, we know well that radicals cause molecular transformations and gene mutations in many types of organisms. Although oxygen is essential for aerobic forms of life, oxygen metabolites are highly toxic. In healthy individuals, free radical production is continuously balanced by natural antioxidative defence systems. Disruption of the balance between reactive oxygen species (ROS) production and elimination, due to, among other things, aging, leads to the process called oxidative stress. As a consequence, ROS are known to be implicated in many cell disorders and in the development of many diseases

including cardiovascular diseases, atherosclerosis, cataracts, chronic inflammation, or neurodegenerative diseases such as Alzheimer's or Parkinson's disease (Gutteridge, 1993; Knight, 1995). Antioxidants, which can inhibit or delay the oxidation of an oxidizable substrate in a chain reaction, therefore, appear to be very important in the prevention of many diseases (Halliwell et al., 1992).

The number of antioxidant compounds synthesized by plants as secondary products, mainly phenolics, serving in plant defence mechanisms to counteract reactive oxygen species (ROS) in order to survive, is currently estimated to be between 4000 and 6000 (Robards et al., 1999;Wollgast & Anklam, 2000; Havsteen, 2002). The antioxidant activities of phenolics are related to a number of different mechanisms, such as free radical-scavenging, hydrogen-donation, singlet oxygen quenching, metal ion chelation, and acting as a substrate for radicals such as superoxide and hydroxyl. A direct relationship has been found between the phenolic content and antioxidant capacity of plants (Robards et al., 1999; Ferreira et al., 2007a). In fact, to counteract deleterious effects of ROS, phenolic compounds, naturally distributed in plants, are effective (Pereira et al. 2006; Ferreira et al., 2007b).

To find new natural sources of active compounds, we studied the antioxidant potential of different constituents of *Castanea sativa* Miller. Among the 12 world chestnuts species, this one is the most consumed being predominant in Portugal, with a relevant place at the socioeconomic level, reaching an annual fruit production of 20000 tons. The best development conditions are found at altitudes higher than 500 m and winter low temperatures, as in the Bragança region (Northeast of Portugal) in which 12,500 ha are used for chestnuts cultivation (Ribeiro et al., 2007). Although it has already been demonstrated that chestnut fruits (Ribeiro et al., 2007) and leaves (Calliste et al., 2005) contain phenolic compounds, little is known about their antioxidant potential and also about other chestnut constituents such as skins and flowers.

2. MATERIALS AND METHODS

2.1. Samples and sample preparation

Chestnut fruits, leaves and flowers from *Cv.* Longal, were collected in August 2006 in a chestnut tree orchard located in Trás-os-Montes, Northeast Portugal. The samples were lyophilized until further use.

A fine dried powder (20 mesh) of sample (5g) was extracted with 50 mL of boiling water for 30 min and filtered through Whatman nº 4 paper. The aqueous extract was frozen, lyophilized, and then redissolved in water at a concentration of 20 mg/mL and stored at 4 ºC for further use.

2.2. Determination of antioxidant contents

Phenolic concentration in the extracts was estimated by a colorimetric assay based on procedures described by Singleton and Rossi (1965) with some modifications. Gallic acid was used for constructing the standard curve (0.01-0.4 mM).

Flavonoids were determined following the procedure described by Jia *et al*. (1999), using (+)-catechin to obtain the standard curve (0.022-0,34 mM).

2.3. Antioxidant activity evaluation assays

The biochemical assays used to screen antioxidant properties were performed according to procedures described by us in previous works (Barros et al., 2007).

DPPH radical scavenging activity was evaluated measuring the absorption at 517 nm due to the reduction of the DPPH radical (% RSA = $[(A_{DPPH}-A_S)/A_{DPPH}] \times 100$, where A_S is the absorbance of the solution when the sample extract has been added at a particular level, and A_{DPPH} is the absorbance of the DPPH solution).

Reducing power was evaluated measuring absorbance at 700 nm after mixing ferric compounds to the samples; a higher absorbance indicates higher reducing power.

The inhibition of β-carotene bleaching was evaluated using the β-carotene-linoleate model system. Lipid peroxidation (β-carotene bleaching in the presence of linoleic acid) inhibition (LPO) was achieved spectrofotometrically at 470 nm and calculated by: LPO inhibition = (β-carotene content after 2h of assay/initial β-carotene content) × 100.

The inhibition of erythrocyte hemolysis mediated by peroxyl free radicals was performed determining the protective effect of the extracts on erythrocyte hemolysis induced by AAPH ((2,2'-azobis(2-amidinopropane)dihydrochloride); % hemolysis inhibition = $[(A_{AAPH}-A_S)/A_{AAPH}] \times 100$, where A_S is the absorbance of the sample containing the extract, and A_{AAPH} is the absorbance of the control sample containing no extract.

The inhibition of lipid peroxidation using brain tissue was measured by the colour intensity of MDA-TBA (malondialdehyde-thiobarbituric acid) complex. MDA, formed from the breakdown of polyunsaturated fatty acid, serves as a convenient index for determining the extent of lipid peroxidation. The inhibition ratio (%) was calculated using the following formula: Inhibition ratio (%) = [(A – B)/A] x 100%, where A and B were the absorbance of the control and the compound solution, respectively.

3. RESULTS AND DISCUSSION

Table 1 presents extraction yields (expressed as w/w percentages), phenol and flavonoids contents obtained for all the chestnut constituents. Despite the low values obtained for the extraction yields, the antioxidants contents found were very good, indicating that the extraction was efficient. Nevertheless, it was not observed a relation between the extracted mass and the corresponding phenols and flavonoids. Probably, fruits and leaves contain higher amounts of other polar compounds, besides the antioxidants quantified in this study, than chestnut flowers and skins. Phenols and flavonoids were found in all the samples and in the order: Outer skin > Inner skin > Flower > Leave >> Fruit.

Table 1. Extraction yields, phenols and flavonoids contents of different chestnut constituents. In each row different letters mean significant differences ($p<0.05$)

Sample	Extraction yield (%)	Phenols (mg/g)	Flavonoids (mg/g)
Flower	16.25	298.18±5.73 c	159.56±5.32 c
Leave	20.91	102.79±2.98 d	54.49±3.74 d
Outer skin	4.98	509.63±18.70 a	502.70±11.21 a
Inner skin	21.57	474.58±27.04 b	329.51±6.13 b
Fruit	19.60	3.73±0.11 e	2.30±0.16 e

Table 2 shows antioxidant activity EC_{50} values of chestnut flowers, leaves, outer and inner skins, and fruits measured by different biochemical assays. Overall, chestnut skins revealed better antioxidant properties (significantly lower EC_{50} values; $p<0.05$). The EC_{50} values obtained for these constituents were excellent (less than 165 μg/mL), particularly for LPO inhibition (less than 12 μg/mL). Chestnut flowers and leaves also revealed very good antioxidant activity, while chestnut fruits presented the higher EC_{50} values in all the tested methods. The obtained results are in agreement with the phenol and flavonoid contents determined for each sample and showed in table 1. The EC_{50} values obtained for lipid peroxidation inhibition were better than for reducing power, scavenging effects on DPPH radicals, β-carotene bleaching inhibition caused by linoleate free radical and for hemolysis inhibition mediated by peroxyl free radicals.

Table 2. EC_{50} values obtained in the antioxidant activity assays of different chestnut constituents. In each line different letters mean significant differences ($p<0.05$)

EC_{50} (µg/mL)	Flower	Leave	Outer skin	Inner skin	Fruit
Scavenging Effect	74.92±0.60 c	169.92±2.49 b	39.66±1.11 d	32.65±0.38 e	> 10000 a
Reducing power	87.29±0.03 c	313.30±0.03 b	55.14±0.05 e	68.66±0.01 d	9043.87±1.48 a
Bleaching inhibition	160.60±17.54 c	1450.22±100.59 b	132.92±10.97 c	163.59±13.84 c	3631.51±283.89 a
Hemolysis inhibition	196.18±6.88 b	169.06±8.99 b	91.35±1.52 c	47.54±0.43 d	3486.48±70.95 a
LPO inhibition	9.93±2.05 c	310.36±11.97 b	7.87±0.15 c	11.54±4.57 c	1116.91±41.04 a

Over two-thirds of cancer-related death could be prevented through lifestyle modification, particularly through dietary means and, chestnut products could contribute to minimize cancer risks trough antioxidants input. Chestnut seems to be a natural provider of antioxidants, increasing its value, since it offers effective protection against oxidative damage, which occurs both in our body and several foods.

In conclusion, the results obtained in this study demonstrate that not only chestnut fruits but also other chestnut constituents may be a good candidate for employment as antioxidants sources. For example, flowers and skins are a good source of healthy compounds, namely phenols and flavonoids, suggesting that they could be useful in the prevention of diseases in which free radicals are implicated. *C. sativa* leaves are already used in folk medicine as a tea to treat hacking cough and diarrhea, and proved to have antibacterial and allelopathic activity (Basile et al., 2000). Nevertheless, to our best knowledge, the present study was the first report to demonstrate that chestnut flowers, skins and fruits have interesting antioxidant potential.

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