

Comparative effects of gamma and electron beam irradiation on the antioxidant potential of Portuguese chestnuts (*Castanea sativa* Mill.)

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Abstract

Chestnuts (*Castanea sativa* Mill.) are widely consumed all over the world, and have been recently studied for their antioxidant potential. The present study reports the effect of e-beam and gamma radiation (doses of 0, 0.5, 1 and 3 kGy) on the antioxidant potential of Portuguese chestnuts. Irradiation might be an alternative preservation method, since Methyl Bromide, a widely used fumigant, was banished by the European Union in 2010 due to its toxicity. The antioxidant activity was evaluated through 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging activity assay, reducing power by the Ferricyanide/Prussian blue assay, and lipid peroxidation inhibition by β -carotene/linoleate and thiobarbituric acid reactive substances (TBARS) assays. The analysis of total phenolics and flavonoids was performed by spectrophotometric assays. Irradiated samples preserved total phenolics content (but not flavonoids) and revealed higher antioxidant activity (lower EC₅₀ values) than the control samples. The most indicated doses to maintain antioxidants content, and to increase antioxidant activity were 1 kGy and 3 kGy for electron beam and gamma radiation, respectively.

Keywords: E-beam, Gamma, Irradiation, Chestnuts, Antioxidants, Phenolics, Flavonoids

1. Introduction

Worldwide chestnut production is estimated to be around 1.1 million tons per year, being China the biggest producer. Europe represents 12% of the world's production, being Italy and Portugal responsible for 4 and 3% respectively. Regarding Portugal, the Trás-os-Montes region is responsible for 82% of the entire chestnut production (Fernandes et al., 2011; Ministério da Agricultura do Desenvolvimento Rural e das Pescas, 2004). The nutritional value of chestnuts produced in the Iberian Peninsula has been extensively studied by various research groups (Barreira et al., 2009; Borges et al., 2008; Pereira-Lorenzo et al., 2006). The antioxidant potential of these nuts has also been reported by Barreira et al. (2008).

Chestnut preservation is extremely important to extend the shelf life and guarantee a pest free fruit. These concerns are even greater when the main objective is exportation. The most common preservation method was fumigation with Methyl Bromide, but since 2010, the European Union banned this chemical, under the Montreal Protocol measures (UNEP, 2006). Since then, many other preservation methods have been tried, such as heat treatment and immersion in water, but they present some disadvantages, like low efficiency, development of moulds and alteration of the chemical composition (Fernandes et al., 2011; Jeremini et al., 2006; UNEP, 2006).

Recently, irradiation has been introduced as an alternative, seen as though it does not have any negative effect on the environment, it reduces the amount of weight loss during post-harvest, doesn't leave any residues on the fruits (Jeremini et al., 2006) and there are no significant changes in the composition of irradiated chestnuts (Antonio et al., 2011; Fernandes et al., 2011). Irradiation in the EU is only allowed with gamma rays, x-rays and electron beam. The maximum limits are 10 kGy for gamma rays, 5

MeV machines for x-rays and 10 MeV for electron beam. Only dried aromatic herbs, spices and vegetable seasonings are allowed to be irradiated in the EU (EU, 1999).

Although many studies should still be carried out, the effect of low doses of gamma irradiation (0.27 ± 0.04 kGy or 0.54 ± 0.04 kGy) on antioxidant potential of chestnuts was already studied, being concluded that the application of gamma irradiation showed to be advantageous for antioxidant activity, independently of the dose used (Antonio et al., 2011). Nevertheless, the storage time was more significant to chestnuts antioxidant potential than the irradiation dose.

Herein, the study was developed using higher doses (0, 0.5, 1 and 3 kGy) and eliminating the storage time effect (all the samples were analyzed immediately after irradiation). Furthermore, electron beam irradiation, a less ionizing radiation, was also tested in order to compare its effects on chestnuts antioxidant potential (free radical scavenging activity, reducing power, lipid peroxidation inhibition, total phenolics and flavonoids) with gamma irradiation.

2. Materials and methods

2.1 Standards and reagents

To prepare the acid aqueous Fricke dosimeter solution the following reagents were used: ferrous ammonium sulfate(II)hexahydrate, sodium chloride and sulfuric acid, all of them purchased from Panreac S.A. (Barcelona, Spain) with purity PA (proanalysis), and water treated in a Milli-Q water purification system (Millipore, model A10, USA). 2,2-Diphenyl-1-picrylhydrazyl (DPPH) was obtained from Alfa Aesar (Ward Hill, MA, USA). The standards trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), gallic acid and (+)-catechin were purchased from Sigma (St. Louis, MO, USA). Methanol and all other chemicals were of analytical grade and obtained from common

sources. Water was treated in a Milli-Q water purification system (TGI Pure Water Systems, USA).

2.2 Samples

Chestnuts (*Castanea sativa* Mill.) samples were obtained from Bragança, Trás-os-Montes (Portugal). For each irradiation procedure, they were divided in four groups: control (non-irradiated, 0 kGy), sample 1 (0.5 kGy), sample 2 (1 kGy), and sample 3 (3 kGy) with fifteen units per group (**Figure 1A**). An independently control was used for each irradiation procedure (gamma and electron beam performed in Portugal and Poland, respectively), in order to guarantee the same conditions for all the samples. Previous to chestnuts irradiation, a dosimetric study was performed using a chemical solution sensitive to ionizing radiation, called Fricke dosimeter.

2.3 Samples irradiation

Gamma radiation. The irradiation of the samples was performed in a Co-60 experimental chamber with four sources, a total activity of 267 TBq (6.35 kCi) in November 2011 (Precisa 22, Graviner Manufacturing Company Ltd, U.K.) (**Figure 1B**). After irradiation geometry dose rate estimation, using the Fricke dosimeter and the procedure described in the standards (ASTM, 1992), the groups for irradiation were placed in Poly(methyl methacrylate) (PMMA) box, or acrylic glass, and irradiated at ambient atmosphere and temperature (15 °C) (**Figure 1C**). During the irradiation process, 4 routine dosimeters were used for each group for the higher dose to monitor the process (Amber Perspex dosimeters, from Harwell Company, U.K.). The samples were rotated up-side down (180°) at half of the time, to increase the dose uniformity. The Amber Perspex dosimeters were read in a UV-VIS Spectrophotometer (Shimadzu

mini UV 1240 spectrophotometer) at 603 nm, two readings for each, to estimate the dose according to a previous calibration curve.

The estimated doses after irradiation were 0.6 ± 0.1 kGy, 1.1 ± 0.1 kGy and 3 ± 0.3 kGy for each of the mentioned groups, respectively, at a dose rate of 0.8 ± 0.1 kGy h⁻¹.

For simplicity, from now on, in the tables and graphs we considered the values 0, 0.5, 1 and 3 kGy, for non-irradiated and irradiated samples.

Electron beam radiation. The irradiation with electrons was performed at the INCT – Institute of Nuclear Chemistry and Technology – in Warsaw, Poland, with an e-beam of 10 MeV of energy (**Figure 1D**). Pulse duration 5.5 μ s, pulse frequency 440 Hz, average beam current 1.1 mA, scan width of 68 cm, conveyer speed in the range 20-100 cm/min, scan frequency 5 Hz. The absorbed dose was 0.53, 0.83 and 2.91 kGy, with an uncertainty of 20% for two first doses and 15% for the last dose. To estimate the dose, Amber Perspex and Gammachrome YR dosimeters (from Harwell Company, U.K.) and a Graphite Calorimeter were used, depending on the dose level. The procedure to read the Amber and Gammachrome YR dosimeters was the one described above. The electrical resistance was read for the calorimeter dosimeter and converted in dose according to a previous calibrated curve.

2.4 Antioxidant activity evaluation

Extraction procedure. After irradiation, all the samples were lyophilized (FreeZone 4.5 model 7750031, Labconco, Kansas, USA), reduced to a fine dried powder (20 mesh) and mixed to obtain homogenate samples. The lyophilized powder (1 g) was stirred with methanol (30 mL) at 25 °C at 150 rpm for 1 h and filtered through Whatman No. 4 paper. The residue was then extracted with an additional portion of methanol. The

combined methanolic extracts were evaporated under reduced pressure (rotary evaporator Büchi R-210; Flawil, Switzerland), re-dissolved in methanol at 10 mg/mL (stock solution), and stored at 4 °C for further use. Successive dilutions were made from the stock solution and submitted to *in vitro* assays already described by the authors (Antonio et al., 2011) to evaluate the antioxidant activity of the samples. The sample concentrations providing 50% of antioxidant activity or 0.5 of absorbance (EC₅₀) were calculated from the graphs of antioxidant activity percentages (DPPH, β-carotene/linoleate and TBARS assays) or absorbance at 690 nm (reducing power assay) against sample concentrations. Trolox was used as standard.

Total phenolics. Phenolics were determined by the Folin–Ciocalteu assay, measuring the absorbance at 765 nm. Gallic acid was used to obtain the standard curve (9.4×10^{-3} - 1.5×10^{-1} mg/mL), and the results were expressed as mg of gallic acid equivalents (GAE) per g of extract.

Total flavonoids. Flavonoids were determined by the AlCl₃ assay, measuring the absorbance at 510 nm. (+)-Catechin was used to calculate the standard curve (4.5×10^{-3} - 2.9×10^{-1} mg/mL) and the results were expressed as mg of (+)-catechin equivalents (CE) per g of extract.

DPPH radical scavenging activity. This methodology was performed by using an ELX800 microplate reader (Bio-Tek Instruments, Inc; Winooski, USA), and calculated as a percentage of DPPH discolouration using the formula: $[(A_{\text{DPPH}} - A_{\text{S}}) / A_{\text{DPPH}}] \times 100$, where A_{S} is the absorbance of the solution containing the sample at 515 nm, and A_{DPPH} is the absorbance of the DPPH solution.

Reducing power. This methodology evaluated the capacity to convert Fe^{3+} into Fe^{2+} , measuring the absorbance at 690 nm in the microplate reader mentioned above.

Inhibition of β -carotene bleaching. This capacity was evaluated through the β -carotene/linoleate assay; the neutralization of linoleate free radicals avoids β -carotene bleaching, which was measured by the formula: $(\beta\text{-carotene absorbance after 2h of assay}/\text{initial absorbance}) \times 100$.

TBARS assay. Lipid peroxidation inhibition in porcine (*Sus scrofa*) brain homogenates was evaluated by the decreasing in thiobarbituric acid reactive substances (TBARS); the colour intensity of the malondialdehyde-thiobarbituric acid (MDA-TBA) was measured by its absorbance at 532 nm; the inhibition ratio (%) was calculated using the following formula: $[(A - B)/A] \times 100\%$, where A and B were the absorbance of the control and the sample solution, respectively.

2.5 Statistical analysis

Three replicates of each sample were used and all the assays were carried out in triplicate. The results are expressed as mean values and standard deviation (SD). The results were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's HSD Test with $\alpha = 0.05$. This analysis was carried out using SPSS v. 18.0 program.

3. Results and discussion

The antioxidant potential was analyzed through total phenolics and flavonoids content, as well as DDPH scavenging activity, reducing power, β -carotene bleaching inhibition and inhibition of lipid peroxidation using thiobarbituric acid reactive substances (TBARS). The results obtained for the samples submitted to electron beam and gamma radiation are presented in **Table 1** and **2**, respectively.

Regarding electron beam radiation, it is clear that the best irradiation dose was 1 kGy, where the samples revealed the highest phenolics content (8.16 mg GAE/g extract) and the highest antioxidant activity (lowest EC_{50} values, 1.66 to 2.81 mg/mL) in general. Flavonoids were affected by electron beam radiation, since the highest level was observed in the control sample (2.34 mg CE/g extract). Therefore, electron beam irradiated samples preserved phenolics since these samples present higher concentrations of the mentioned compounds than the control sample (non-irradiated) (**Table 1**). Moreover, all the irradiated samples (0.5, 1 and 3 kGy) revealed higher antioxidant activity (lower EC_{50} values) than the control sample in all the assayed methods.

Concerning gamma radiation, it is clear that the best dose was 3 kGy; those samples proved to have the highest content in phenolics (5.55 mg GAE/g extract; without statistical significant differences in relation to samples irradiated with 1 kGy) and the lowest EC_{50} values for DPPH scavenging activity, β -carotene bleaching and TBARS inhibition (the last one also without statistical significant differences in relation to samples irradiated with 1 kGy). Otherwise, control samples gave the highest flavonoids content (1.21 mg CE/g extract), and the highest reducing power (lowest EC_{50} value; 2.81 mg/mL). The obtained results are in agreement with a previous study in chestnuts irradiated with low doses of gamma radiation (≤ 0.5 kGy), where it was concluded that the application of gamma irradiation showed to be advantageous for antioxidant

activity, independently of the dose used ([Antonio et al., 2011](#)). Furthermore, the control samples (especially in electron beam irradiation) revealed very close EC₅₀ values (DDPH scavenging activity, reducing power and β-carotene bleaching inhibition) to a previous study in non-irradiated chestnuts ([Barreira et al., 2008](#)).

Figure 2 represents individually the results obtained in each antioxidant activity assay for the best dose of each radiation type (1 and 3 kGy for electron beam and gamma radiation, respectively), in comparison with control samples. Irradiated samples showed higher DPPH scavenging activity, reducing power and β-carotene bleaching inhibition than control samples. Chestnuts irradiated with gamma irradiation at 3 kGy revealed the highest DPPH scavenging activity (**Figure 2A**), while chestnuts irradiated with electron beam radiation at 1 kGy revealed the highest reducing power (**Figure 2B**), β-carotene bleaching inhibition and TBARS inhibition (**Figure 2C**).

The control samples of both radiations types (gamma and electron beam) correlated quite well with flavonoids, displaying a R² of 0.9430, 0.7909 and 0.7426 for DPPH scavenging activity, reducing power and TBARS inhibition, respectively. These results are once more in agreement with the results published by [Barreira et al., \(2008\)](#), proving that flavonoids are a group of polyphenols that contribute in a great manner for chestnuts antioxidant activity. In fact, these phenolic compounds are widely found in chestnuts ([Dinis et al., 2012](#)) and, in some cases represent an average of 0.88% of their dry weight ([Kapusta et al., 2007](#)). Flavonoids, are proven to be the major contributor to the scavenging of reactive oxygen species and to have a potent cell-protective effect ([Masaki et al., 1995](#)). Nevertheless, the correlations between antioxidant activity of irradiated samples and flavonoids decreased drastically (R²<3.00) revealing that these compounds may be sensitive to both electron beam and gamma radiation (indicated by the decrease in flavonoids content observed in irradiated samples). Despite the decrease

in flavonoids (a class of phenolic compounds), total phenolics increased which could be responsible for the increasing in antioxidant potential of irradiated samples. Therefore, it can be concluded that other phenolic compounds but not flavonoids contribute more for antioxidant activity.

Overall, irradiated samples preserved total phenolics content (certainly other phenolic compounds rather than flavonoids) and revealed higher antioxidant activity (lower EC₅₀ values) than the control samples. The most indicated doses to maintain antioxidants content, and to increase antioxidant activity were 1 kGy and 3 kGy for electron beam and gamma radiation, respectively. Future studies should be performed in order to evaluate the effects of irradiation in individual phenolic compounds, using chromatographic techniques.

The authors declare that they not have any conflict of interest.

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Table 1. Phenolics (mg GAE/g extract), flavonoids (mg CE/g extract) and antioxidant activity (EC₅₀ values, mg/mL) of chestnuts submitted to electron beam irradiation.

Mean±SD (n=9).

	Control	0.5 kGy	1 kGy	3 kGy
Phenolics	3.61 ± 0.57 ^d	4.06 ± 0.93 ^c	8.16 ± 0.34 ^a	5.60 ± 0.50 ^b
Flavonoids	2.34 ± 0.25 ^a	0.40 ± 0.05 ^b	0.31 ± 0.06 ^c	0.24 ± 0.06 ^c
DPPH scavenging activity	25.12 ± 1.11 ^a	23.27 ± 2.61 ^b	15.93 ± 0.71 ^c	13.81 ± 1.67 ^d
Reducing power	7.05 ± 0.96 ^a	6.31 ± 0.59 ^b	2.81 ± 0.10 ^d	5.36 ± 0.27 ^c
β-carotene bleaching inhibition	6.00 ± 0.53 ^b	2.54 ± 0.37 ^c	1.94 ± 0.20 ^d	6.95 ± 1.09 ^a
TBARS inhibition	10.63 ± 1.72 ^a	4.06 ± 1.28 ^c	1.66 ± 0.41 ^d	7.82 ± 2.77 ^b

In each row, different letters mean significant differences between doses ($p < 0.05$).

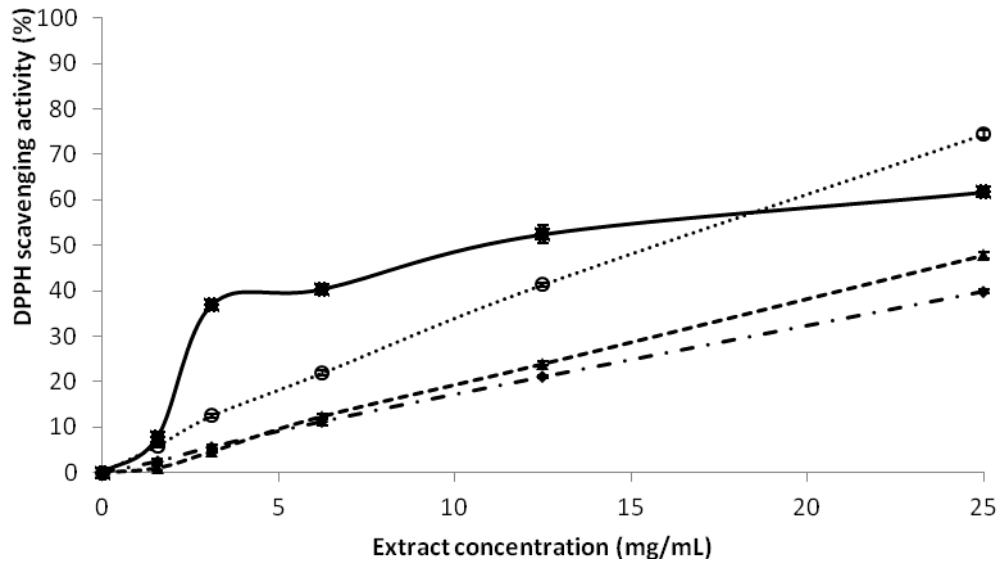
Concerning the antioxidant activity assays, the results are presented in EC₅₀ values, what means that higher values correspond to lower reducing power or antioxidant potential. EC₅₀: Extract concentration corresponding to 50% of antioxidant activity or 0.5 of absorbance for the reducing power assay.

Table 2. Phenolics (mg GAE/g extract), flavonoids (mg CE/g extract) and antioxidant activity (EC₅₀ values, mg/mL) of chestnuts submitted to gamma irradiation. Mean±SD (n=9).

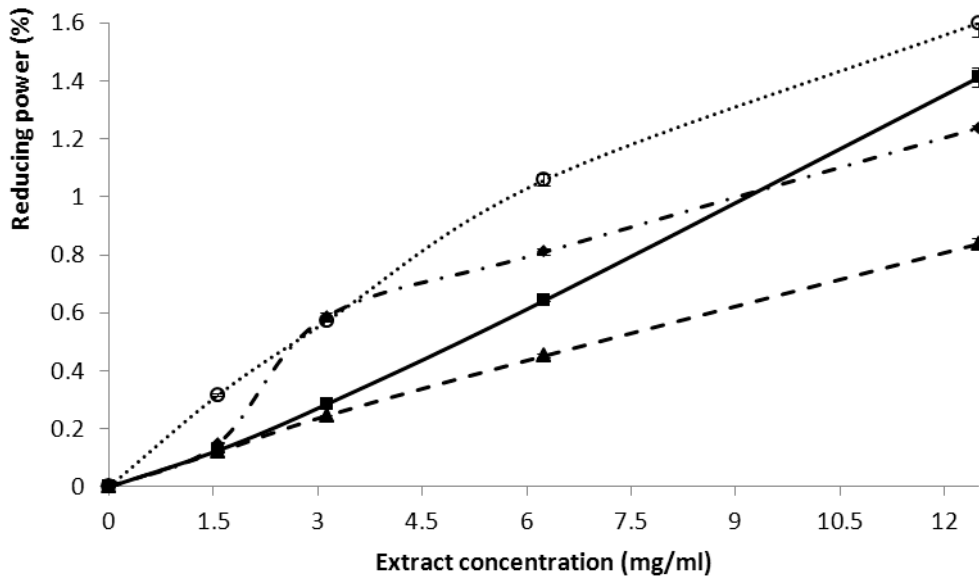
	Control	0.5 kGy	1 kGy	3 kGy
Phenolics	3.63 ± 0.01 ^c	4.26 ± 0.44 ^b	5.07 ± 0.42 ^a	5.55 ± 1.21 ^a
Flavonoids	1.21 ± 0.00 ^a	1.15 ± 0.11 ^a	0.42 ± 0.04 ^b	0.55 ± 0.05 ^b
DPPH scavenging activity	38.72 ± 0.85 ^b	45.48 ± 2.43 ^a	15.05 ± 0.94 ^c	11.30 ± 0.92 ^d
Reducing power	2.81 ± 0.03 ^c	5.30 ± 0.62 ^a	5.45 ± 0.73 ^a	4.05 ± 0.69 ^b
β-carotene bleaching inhibition	6.38 ± 0.66 ^b	9.23 ± 1.31 ^a	3.60 ± 0.54 ^c	2.51 ± 0.37 ^d
TBARS inhibition	5.21 ± 0.36 ^b	10.22 ± 1.29 ^a	1.09 ± 0.42 ^c	0.86 ± 0.21 ^c



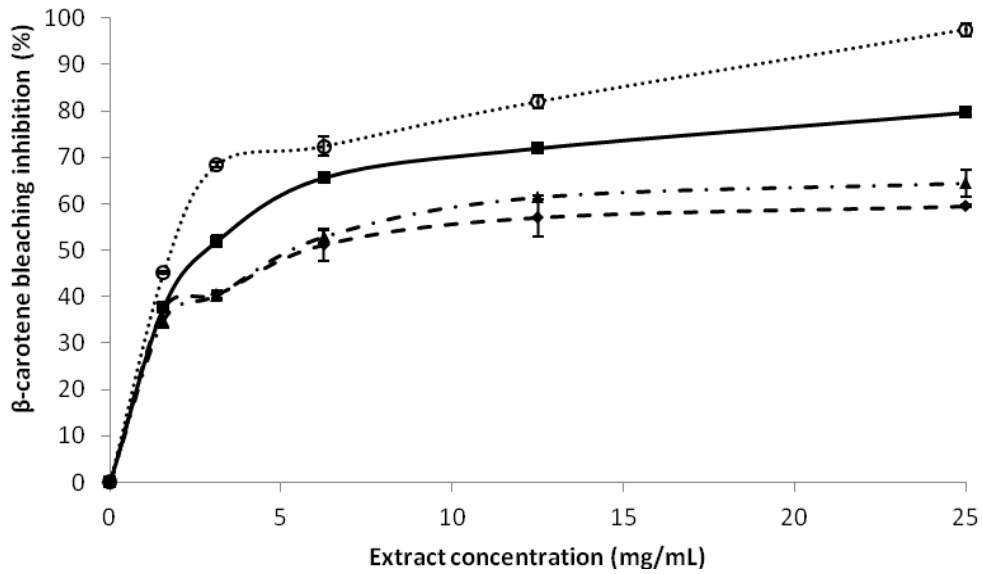
Figure 1. A. Chestnuts after electron beam irradiation, from left to right: Control (0 kGy), 0.5 kGy, 1 kGy and 3 kGy. B. Gamma irradiation chamber. C. Chestnuts with corresponding dosimeter inside a Poly(methyl methacrylate) box before gamma irradiation. D. Chestnuts with corresponding dosimeters before electron beam irradiation.



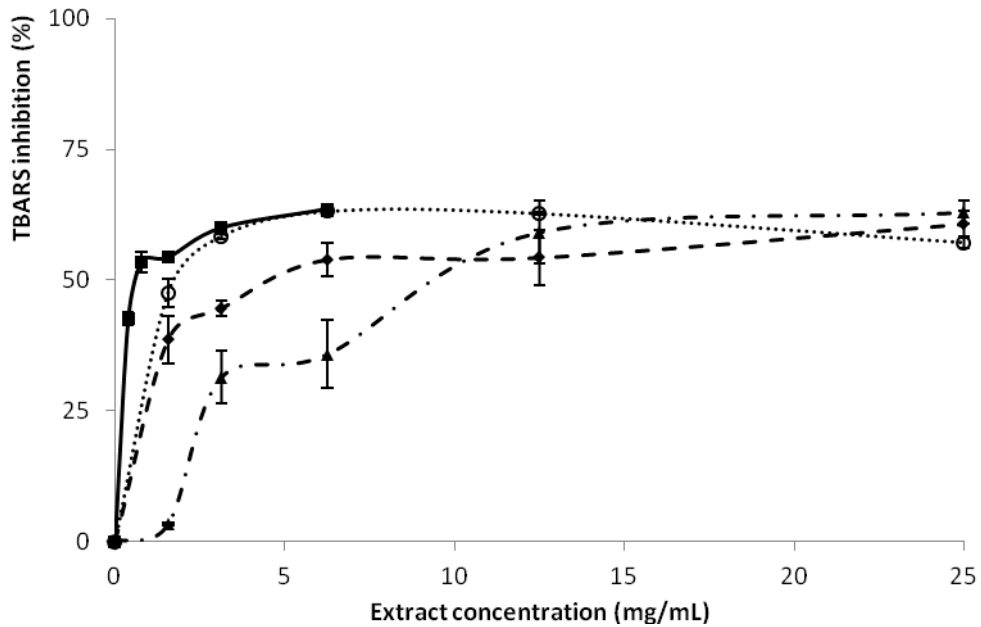
A



B



C



D

Figure 2. DPPH scavenging activity (A), reducing power (B), β -carotene bleaching inhibition (C) and TBARS inhibition (D) of chestnut samples (Mean \pm SE; n = 9): submitted to electron beam- control (- ▲ -) and 1 kGy ($\cdots\circ\cdots$); submitted to gamma radiation- control (- ◆ -) and 3 kGy (—■—).